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(54) Expression of biologically active PDGF analogs in eucaryotic cells.

(57) Methods for expressing a variety of biologically active PDGF analogs in eucaryotic cells are disclosed. The methods generally comprise introducing into a eucaryotic host cell a DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells. The DNA construct contains a transcriptional promoter followed downstream by a suitable DNA sequence. The DNA sequence may encode a protein substantially homologous or identical to the A-chain or the B-chain of PDGF, or a portion thereof, or an A-B heterodimer. In addition, a portion of the DNA sequence may encode at least a portion of the A-chain, while another portion encodes at least a portion of the B-chain of PDGF. Eucaryotic cells transformed with these DNA constructs are also disclosed. Methods of promoting the growth of mammalian cells, comprising incubating the cells with a biologically active PDGF analog expressed by a eucaryotic host cell transformed with such a DNA construct, are also disclosed.

Dimeric proteins having substantially the same biological activity as PDGF are also disclosed. More specifically, the proteins may have two polypeptide chains, one of the chains being a mosaic of amino acid sequences substantially identical to portions of the A- or B-chains of PDGF, the second of the chains being substantially homologous to either the A-chain or the B-chain of PDGF, or each of the chains may be substantially identical to the A-chain of PDGF. Alternatively, each of the two polypeptide chains may be a mosaic of amino acid sequences as described above. In addition, proteins comprising polypeptides that are variants or derivatives of the A- or B-chains of PDGF are also disclosed. Therapeutic compositions containing these proteins and methods for enhancing the wound-healing process in warm-blooded animals are also disclosed.

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Description

EXPRESSION OF BIOLOGICALLY ACTIVE PDGF ANALOGS IN EUCARYOTIC CELLS

Technical Field

The present invention relates to the production of PDGF analogs in general, and more specifically, to the expression of biologically active PDGF analogs in eucaryotes.

Background Art

Human platelet-derived growth factor (PDGF) has been shown to be the major mitogenic protein in serum for mesenchymal derived cells. This is well documented by numerous studies of platelet extracts or purified PDGF induction of either cell multiplication or DNA synthesis (a prerequisite for cell division) in cultured smooth muscle cells, fibroblasts and glial cells (Ross et al., PNAS 71: 1207, 1974; Kohler and Lipton, Exp. Cell Res. 87: 297,1974; Westermark and Wasteson, Exp. Cell Res. 98: 170, 1976; Heldin et al., J. Cell Physiol. 105: 235, 1980; Raines and Ross, J. Biol. Chem. 257: 5154, 1982). Furthermore, PDGF is a potent chemoattractant for cells that are responsive to it as a mitogen (Grotendorst et al., J. Cell Physiol. 113: 261, 1982; Seppa et al., J. Cell Biol. 92: 584, 1982). It is not generally the case that mitogens also act as chemotactic agents. Due to its mitogenic activity, PDGF is useful as an important component of a defined medium for the growth of mammalian cells in culture, making it a valuable research reagent with multiple applications in the study of animal cell biology.

In vivo, PDGF normally circulates stored in the alpha granules of platelets. Injury to arterial endothelial linings causes platelets to adhere to the exposed connective tissue and release their granules. The released PDGF is thought to chemotactically attract fibroblasts and

smooth muscle cells to the site of injury and to induce their focal proliferation as part of the process of wound repair (Ross and Glomset, N. Eng. J. of Med. 295: 369, 1976).

It has been postulated that as a part of this response to injury, PDGF released by platelets may play a causative role in the development of the proliferative lesions of atherosclerosis (Ross and Glomset, ibid.) which is one of the principal causes of myocardial and cerebral infarction. Strategies for the prophylaxis and treatment of atherogenesis in the past have been narrowly directed toward reducing risk factors for the disease, such as lowering blood pressure in hypertensive subjects and reducing elevated cholesterol levels in hypercholesterolemic subjects.

Recent studies have shown that at least one of the two protein chains comprising PDGF and the putative transforming protein of simian sarcoma virus (SSV), an acute transforming retrovirus, appear to have arisen from the same or closely related cellular genes. In particular, computer analysis of a partial amino acid sequence of PDGF has revealed extensive homology with the gene product, $p28^{sis}$, of SSV (Doolittle et al., Science 221: 275, 1983; Waterfield et al., Nature 304: 35, 1984; and Johnson et al., EMBO J. 3: 921, 1984). Further, more recent studies have illustrated that $p28^{sis}$ and PDGF show antigenic as well as structural similarities (Robbins et al., Nature 305: 605, 1983; Niman, Nature 307: 180, 1984).

Although previous attempts, such as that summarized in Devare et al. (Cell 36: 43, 1984), have been made to express the v-sis gene in a transformed microorganism, they have not been successful in producing mitogenic material. More recently, investigators have described the production of $p28^{sis}$ in E. coli as a fusion protein (Wang et al., J. Biol. Chem. 259: 10645, 1984). This protein appears to compete with PDGF for binding to PDGF receptor sites. While SSV transformed rodent cells have been shown

to exhibit a mitogenic activity similar to PDGF (Deuel et al., Science 221: 1348, 1983; Owen et al., Science 225: 54, 1984), it is not clear that this activity is due to a gene product from SSV (i.e., $p28^{sis}$). Furthermore, cells transformed by a variety of viruses other than SSV produce a PDGF-like mitogen into the culture medium (Bowen-Pope et al., PNAS 81: 2396, 1984).

While natural PDGF may be isolated from human plasma or platelets as starting material, it is a complex and expensive process, in part due to the limited availability of the starting material. In addition, it is difficult to purify PDGF with high yield from other serum components due to its extremely low abundance and biochemical properties. Furthermore, the therapeutic use of products derived from human blood carries the risk of disease transmission due to contamination by, for example, hepatitis virus, cytomegalovirus, or HIV.

In view of PDGF's clinical applicability in the treatment of injuries in which healing requires the proliferation of fibroblasts or smooth muscle cells and its value as an important component of a defined medium for the growth of mammalian cells in culture, the production of useful quantities of protein molecules similar to authentic PDGF which possess mitogenic activity is clearly invaluable.

In addition, the ability to produce relatively large amounts of PDGF or PDGF analogs would be a useful tool for elucidating the putative role of the v-sis protein, $p28^{sis}$, in the neoplastic process.

Further, since local accumulation of smooth muscle cells in the intamal layer of an arterial wall is central to the development of atherosclerotic lesions (Ross and Glomset, ibid.), one strategy for the prophylaxis and treatment of atherosclerosis would be to suppress smooth muscle cell proliferation. The ability to produce large amounts of PDGF would be useful in developing inhibitors or designing specific approaches which prevent or interfere

with the in vivo activity of PDGF in individuals with atherosclerosis.

Disclosure of the Invention

Briefly stated, the present invention discloses methods for expressing a variety of biologically active PDGF analogs in eucaryotic cells. In general, the methods comprise introducing into a eucaryotic host cell a DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells. The DNA construct contains a transcriptional promoter followed downstream by an appropriate DNA sequence.

In one aspect of the present invention, the DNA sequence encodes a polypeptide which is substantially homologous to the A-chain of PDGF. In another aspect of the present invention, the DNA sequence encodes a polypeptide which is substantially homologous to the B-chain of PDGF. Within a third aspect of the present invention, a portion of the DNA sequence encodes a polypeptide which is substantially homologous to at least a portion of the A-chain of PDGF, and another portion of said DNA sequence encodes a polypeptide which is substantially homologous to at least a portion of the B-chain of PDGF, these portions of the DNA sequence encoding a protein having substantially the same biological activity as PDGF.

In yet another aspect of the present invention, the DNA construct contains a transcriptional promoter followed downstream by a DNA sequence encoding a polypeptide chain substantially homologous to the A-chain of PDGF, and a transcriptional promoter followed downstream by a DNA sequence encoding a polypeptide chain substantially homologous to the B-chain of PDGF, the chains forming a heterodimer. The protein products produced by the methods utilizing these and other DNA sequences are also disclosed.

The present invention also discloses a variety of other DNA constructs capable of directing the expression.

and secretion of biologically active PDGF analogs in eucaryotic cells. The DNA constructs contain a transcriptional promoter followed downstream by a suitable DNA sequence. As noted above, suitable DNA sequences include those encoding a protein which is substantially homologous to the A-chain or B-chain of PDGF. In addition, the DNA sequence may include a portion encoding a polypeptide which is substantially homologous to at least a portion of the A-chain of PDGF, and a portion encoding a polypeptide which is substantially homologous to at least a portion of the B-chain of PDGF. Further, the DNA construct may contain transcriptional promoters followed downstream by DNA sequences encoding polypeptide chains substantially homologous to the A- and B-chains of PDGF, the chains forming a heterodimer.

Eucaryotic host cells transformed with DNA constructs, such as those described above, are also disclosed. A preferred eucaryotic host cell in this regard is a yeast cell.

Another aspect of the present invention discloses methods of promoting the growth of mammalian cells, comprising incubating the cells with a biologically active PDGF analog expressed by a eucaryotic host cell transformed with a DNA construct as described above, and a signal sequence capable of directing the secretion of the protein from the eucaryotic cell.

In another aspect of the present invention, a protein is disclosed having two polypeptide chains, each of said chains being substantially homologous to the A-chain of PDGF. The polypeptide chains may also be substantially identical to the A-chain of PDGF. For purposes of the present invention, "substantially identical polypeptide chains" are those chains that are at least eighty percent homologous to one another at the amino acid level. Within the present invention, the phrase "substantially homologous" refers to those sequences that are at least 30% homologous to one another.

In yet another aspect of the present invention, a protein is disclosed having two polypeptide chains, one of the chains being a mosaic of amino acid sequences substantially identical to portions of the A- or B-chains of PDGF, the second of the chains being substantially homologous to the A- or B-chain of PDGF, the protein having substantially the same biological activity as PDGF. The polypeptide chains may also be substantially identical to one another. Alternatively, the protein may be composed of two polypeptide chains, each of the chains being a mosaic of amino acid sequences substantially identical to the A- or B-chains of PDGF, the protein having substantially the same biological activity as PDGF.

In addition, proteins comprising polypeptides that are variants and derivatives of the A-chain and B-chain of PDGF are also disclosed. These proteins include both homodimers and heterodimers. These modifications to the A-chain and B-chain fall basically into two broad classes, amino acid deletions and amino acid substitutions.

Preferred amino acid substitutions include the replacement of selected cysteine residues with another amino acid, as well as the replacement of other amino acids, the substitution of which does not destroy the biological activity of the resultant molecule. In particular embodiments of the present invention, proteins are disclosed that include the substitution of A-chain cysteine residue at position 10 or B-chain cysteine residue at position 16. Other preferred amino acid substitutions include the replacement of a B-chain phenylalanine residue with a tyrosine residue.

In regard to amino acid deletions, polypeptide chains are disclosed that are substantially identical to the A-chain of PDGF from (a) amino acid 9 to amino acid 104; (b) amino acid 23 to amino acid 104; (c) amino acid 9 to amino acid 95; (d) amino acid 23 to amino acid 95; or (e) amino acid 1 to amino acid 95, the A-chain itself consisting of amino acids 1 to 104. In addition, polypetide

chains are disclosed that are substantially identical to the B-chain of PDGF from (a) amino acid 15 to amino acid 109; (b) amino acid 29 to amino acid 109; (c) amino acid 15 to amino acid 101; (d) amino acid 29 to amino acid 101; or (3) amino acid 1 to amino acid 101, the B-chain itself consisting of amino acids 1 to 109. Removal of amino- and/or carboxy-terminal amino acids as described herein results in smaller biologically active molecules which may have broader therapeutic utility. In addition, the protein described above may have the amino acid sequence of Figure 9, from A-chain amino acid 1 to amino acid 104, or from B-chain amino acid 1 to amino acid 109.

In another aspect of the present invention, a therapeutic composition is disclosed comprising a protein having two substantially identical polypeptide chains, each of said chains being substantially homologous to the A-chain of PDGF, and a physiologically acceptable carrier or diluent. As noted above, the polypeptide chains may also be substantially identical to the A-chain of PDGF. In addition, proteins comprising variants and derivatives of the A-chain of PDGF as described above are also suitable for use in the therapeutic compositions of the present invention.

In still another aspect of the present invention, a therapeutic composition is disclosed comprising a protein having two polypeptide chains, one of the chains being a mosaic of amino acid sequences substantially identical to portions of the A- or B-chains of PDGF, the second of the chains being substantially homologous to the A- or B-chain of PDGF, the protein having substantially the same biological activity as PDGF, and a physiologically acceptable carrier or diluent. Alternatively, the protein may be composed of two polypeptide chains, each of the chains being a mosaic of amino acid sequences substantially identical to the A- or B-chains of PDGF, the protein having substantially the same biological activity as PDGF. As noted above, the polypeptide chains may also be substantially

identical to one another. In addition, proteins comprising variants and derivatives of the A-chain and B-chain of PDGF as described above are also suitable for use in the therapeutic compositions of the present invention.

A related aspect of the present invention is directed toward a method for enhancing the wound-healing process in warm-blooded animals. The method generally comprises administering to the animal a therapeutically effective amount of one or more of the proteins described above, and a physiologically acceptable carrier or diluent.

Other aspects of the invention will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1A is a schematic restriction map of the proviral genome of SSV.

Figure 1B depicts the nucleotide sequence and predicted amino acid sequence encoded by the v-sis region of the SSV genome.

Figure 2 illustrates the construction of a plasmid which contains the MFα1 promoter and secretory signal sequence upstream of the v-sis gene.

Figure 3 illustrates the construction of plasmid p192.

Figure 4 illustrates the oligonucleotide-directed deletion mutagenesis of the amino terminal 66 v-sis codons.

Figure 5 illustrates the construction of plasmid p270.

Figure 6 illustrates the insertion of v-sis expression units upstream of the TPI1 terminator.

Figure 7 illustrates the construction of plasmid pTVS2αT.

Figure 8 illustrates the construction of a B-chain expression unit VSB and its introduction into the pMPOT2 vector.

Figure 9 depicts the amino acid sequences of the mature A- and B-chains of PDGF.

Figure 10 is a dose response curve of PDGF receptor binding by media concentrates from yeast transformants containing plasmids pVSBm and pMPOT2 compared to authentic PDGF.

Best Mode For Carrying Out the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

Polypeptide: A polymer of amino acids.

Reading Frame: The arrangement of nucleotide codons which encode an uninterrupted stretch of amino acids. During translation of an mRNA, the proper reading frame must be maintained. For example, the sequence GCUGGUUGUAAG may be translated into three reading frames or phases, depending on whether one starts with G, with C, or with U, and thus may yield three different peptide products. Translation of the template begins with an AUG codon, continues with codons for specific amino acids, and terminates with one of the translation termination codons.

Coding Sequence: DNA sequences which in the appropriate reading frame directly code for the amino acids of a protein.

Complementary DNA: or cDNA. A DNA molecule or sequence which has been enzymatically synthesized from the sequences present in an mRNA template, or a clone of such a molecule.

Secretory Signal Sequence: That portion of a gene or cDNA encoding a signal peptide. A signal peptide is the amino acid sequence in a secretory protein which

signals its translocation into the secretory pathway of the cell. Signal peptides generally occur at the beginning (amino terminus) of the protein and are approximately 20-40 amino acids long with a stretch of about 9-10 hydrophobic amino acids near the center. Very often the signal sequence is proteolytically cleaved from the protein during the process of secretion.

Cell Surface Receptor: A protein molecule at the surface of a cell which specifically interacts with or binds a molecule approaching the cell's surface. Once the receptor has bound the cognate molecule, it effects specific changes in the physiology of the cell.

Mitogen: A molecule which stimulates cells to undergo mitosis. Mitosis is asexual somatic cell division leading to two daughter cells, each having the same number of chromosomes as the parent cell.

Transformation: The process of stably and hereditably altering the genotype of a recipient cell or microorganism by the introduction of purified DNA. This is typically detected by a change in the phenotype of the recipient organism.

Transcription: The process of producing a mRNA template from a structural gene.

Expression: The process, starting with a structural gene or cDNA, of producing its polypeptide, being a combination of transcription and translation. An expression vector is a plasmid-derived construction designed to enable the expression of a gene or cDNA carried on the vector.

Plasmid: An extrachromosomal, double-stranded DNA sequence comprising an intact "replicon" such that the

plasmid is replicated in a host cell. When the plasmid is placed within a unicellular organism, the characteristics of that organism may be changed or transformed as a result of the expression of the DNA sequences of the plasmid. For example, a plasmid carrying the gene for tetracycline resistance (tet^R) transforms a cell previously sensitive to tetracycline into one which is resistant to it.

Yeast Promoter: DNA sequences upstream from a yeast gene which promote its transcription.

Biological Activity: Some function or set of activities performed by a molecule in a biological context (i.e., in an organism or an in vitro facsimile). In the case of PDGF, these biological activities include the induction of chemotaxis and/or mitogenesis of responsive cell types, following the binding of PDGF to specific cell surface receptors. Other biological effects of human platelet PDGF may include: phospholipase activation; increased phosphatidylinositol turnover and prostaglandin metabolism; stimulation of both collagen and collagenase synthesis by responsive cells; an indirect proliferative response of cells lacking PDGF receptors; and potent vasoconstrictor activity.

PDGF Analog: A polypeptide which is substantially homologous to at least a portion of the A-chain or the B-chain of PDGF, or both, wherein the polypeptide exhibits biological activity as defined herein.

As noted above, human platelet-derived growth factor (PDGF) has been shown to be a major mitogen in serum. PDGF, as it is isolated from platelets, is a different molecule from the novel proteins of the present invention. PDGF is known to be composed of two polypeptide chains, an A-chain and a B-chain, which are held together by disulfide bonds to form the biologically active heterodimer molecule.

This structure has been confirmed by immunoprecipitation experiments (Hart et al., Heldin et al., unpublished). These investigators used monoclonal antibodies directed specifically against the A-chain or the B-chain to immunoprecipitate PDGF. Their results indicate that the PDGF can be removed from solution with antibodies which recognize either chain alone. This confirms the structure of PDGF as a heterodimer of two different polypeptide chains. In addition, naturally-occurring PDGF contains carbohydrate (Deuel et al., J. Biol. Chem. 256: 8896-8899, 1981). Following complete chemical reduction, the single polypeptide chains alone do not exhibit any mitogenic activity (Raines and Ross, ibid.), and attempts to reconstitute activity by reoxidation of the reduced polypeptides have not been successful. Recently, the amino acid sequence of the B-chain has been determined and shown to be substantially homologous to a portion of the v-sis gene product, $p28^{sis}$ (Doolittle et al., ibid.; Waterfield et al., ibid.; and Johnson et al., ibid.). The homology between these two proteins strongly suggests that they are derived from the same or closely related cellular genes.

Given the fact that a single reduced A-chain or B-chain polypeptide is not biologically active and that previous attempts directed toward expressing v-sis sequences in E. coli did not yield mitogenic material, it would not be expected that merely expressing a sequence encoding a PDGF-like molecule in a microorganism would result in a molecule which exhibited biological activity.

The present invention, however, unlike the previous attempts noted above, unexpectedly provides for the expression of DNA sequences encoding PDGF A-chain or B-chain, variants or derivatives of the A- and B-chains, as well as mosaics of portions of the A- and B-chains or their derivatives, in a manner that the expressed molecules exhibit biological activity characteristic of PDGF. Further, the expression system of the present invention was designed to produce the gene product via a eucaryotic secretory path-

way. This enables the expressed polypeptide molecules to be properly processed, correctly folded and assembled into biologically active dimers. Indeed, the present invention, in contrast to previous efforts, results in the secretion of PDGF-like dimers which are biologically active in established assays for PDGF activity, i.e., radioreceptor assay (RRA), mitogenesis assay, and chemotaxis assay.

In its biologically active form, PDGF is a heat-stable protein composed of heterogeneously sized species ranging between 28,000 and 31,000 Daltons, all of the individual species being active in stimulating DNA synthesis (Raines and Ross, ibid.; Deuel et al., J. Biol. Chem. 256: 8896, 1981; Antoniades, PNAS 78: 7314, 1981). Where individual species with molecular sizes of 27,000; 28,500; 29,000; and 31,000 Daltons have been isolated and analyzed, they show extensive tryptic peptide homology and have been found to have comparable mitogenic activity and amino acid composition (Raines and Ross, ibid.). The slight variations in size among the species are most probably due to differences in carbohydrate composition and minor proteolysis.

Through studies of PDGF which has been extensively purified from platelet-rich human plasma, it is likely, as noted above, that PDGF is composed of two polypeptide chains, an A-chain (14,000 Daltons) and a B-chain (16,000 Daltons), which are disulfide bonded together to form the biologically active dimer molecule (Raines and Ross; Deuel et al.; Antoniades, ibid.). The PDGF nomenclature found in the literature is not consistent (Doolittle et al.; Waterfield et al.; Raines and Ross; Johnsson et al., ibid.). The nomenclature of Johnsson et al. (ibid.), wherein the two polypeptides found in pure PDGF are called "A-chain" and "B-chain," is adopted herein. The B-chain is homologous to $p28^{sis}$ and was previously called "peptide-I" (Waterfield et al., ibid.) or "1a" (Doolittle et al., ibid.). The A-chain was previously termed "peptide II" (Waterfield et al., ibid.) or "2a" (Doolittle et al., ibid.). Data

derived from a partial amino acid sequence of PDGF indicate that the two polypeptide chains (A-chain and B-chain) show extensive homology (Doolittle et al., ibid.; Waterfield et al., ibid.; and Johnsson et al., ibid.; Antoniades and Hunkapiller, Science 220: 963, 1983). More specifically, it has been reported that there is 56% amino acid identity between the two chains. In addition, as shown in Figure 9, there are several blocks of perfect homology between the two chains. Further, both of the chains contain eight cysteine residues at identical positions, suggesting that each polypeptide folds into a similar three-dimensional structure.

It appears that these two polypeptides are closely related members of a small family. The blocks of perfect homology between the A- and B-chains reflect regions of the protein which may contribute to function, while the less homologous regions may reflect portions of the protein which are less important to its function. Therefore, as further exemplified by the present invention, certain portions of either the A- or B-chains may be deleted or substituted, while retaining biological activity within the resultant protein.

Based upon the teachings of the present invention, the homology between the A- and B-chains, together with the coincidence of cysteine residues, one skilled in the art can design additional suitable members of this homologous family. For example, one skilled in the art could construct a variety of hybrids between the A- and B-chain genes which would encode proteins in which the homologous domain structures were preserved. It is demonstrated herein that these proteins can be expected to assume a three-dimensional structure similar to wild-type A- or B-chains and retain biological activity.

The v-sis gene, as mentioned above, is the transforming gene of simian sarcoma virus (SSV). The v-sis gene has been cloned and its DNA sequence determined (Devare et al., PNAS 79: 3179, 1982; Devare et al., PNAS 80: 731,

1983). Analysis of this sequence revealed an open reading frame which could encode a 28,000 Dalton protein, designated $p28^{sis}$. Subsequently, such a protein was immunologically identified in SSV-infected cells (Niman, ibid.; Robbins, ibid.). The predicted amino acid sequence of the v-sis gene product, $p28^{sis}$, was found to have a high degree of homology with the actual amino acid sequence of a portion of the B-chain of PDGF (Johnsson, ibid.). The homology of the PDGF B-chain to the v-sis gene product begins at amino acid 67 of $p28^{sis}$, a serine, and continues for 109 amino acids to a threonine residue at amino acid 175. The amino acid sequences preceding and following the B-chain homologous region of $p28^{sis}$ are not homologous to either the A- or B-chains of mature PDGF (Johnsson, ibid.) and represent portions of the B-chain precursor. In addition, PDGF and $p28^{sis}$ have been shown to be similar antigenically (Niman, ibid.; Robbins, ibid.). The v-sis gene product, $p28^{sis}$, a protein of approximately 226 amino acids, dimerizes and is proteolytically processed to a protein of approximately 20,000 Daltons ($p20^{sis}$) in SSV infected cells (Niman, ibid.; Robbins, ibid.). This 20,000 Dalton protein can be immunoprecipitated with antiserum against PDGF.

The mature B-chain homologous region of v-sis encodes a 109 amino acid polypeptide which is almost identical to the human B-chain. The four amino acid differences between these two gene products occur at positions 6, 7, 91 and 97. The mature human A-chain sequence is 104 amino acids in length, and is 56 percent homologous to the B-chain, therefore having a degree of homology to the v-sis product similar to its homology to the B-chain.

In contrast to naturally-occurring PDGF, one particular aspect of this present invention discloses protein products that are disulfide-bonded dimers of two A-chain-like polypeptides. One such dimer, comprising chains having complete homology to the 104 amino acids of PDGF A-chain, migrates on polyacrylamide gels with an

apparent molecular weight of ca. 31,000 Daltons. When the dimer is chemically reduced, the component chains migrate to a position consistent with a polypeptide of 104 amino acids. The amino acid composition of the pure protein has been determined and the results show that the composition is substantially identical to the A-chain sequence shown in Figure 9. The amino acid sequence of this pure, yeast-expressed protein was determined using a gas-phase sequenator (Applied Biosystems). All of the amino terminal sequence obtained could be accounted for by the sequence information shown for the A-chain in Figure 9. These results indicate that the proteins of this aspect of the present invention are homodimers consisting of polypeptide chains homologous to the A-chain of PDGF. The amino acid sequence of the A-chain produced in yeast contained no N-linked glycosylation sites. The glycosylation site present in the native human A-chain was purposely omitted from this construction in order to avoid yeast carbohydrate addition. There is no evidence, based on polyacrylamide gel electrophoresis, that the yeast-expressed A-chain contains carbohydrate. The biological activity observed for these unglycosylated PDGF analogs is somewhat surprising in view of the dependence on glycosylation for biological activity associated with several glycoprotein hormones and other growth factors.

As noted above, another aspect of the present invention discloses proteins comprising polypeptides which are variants and derivatives of the A-chain or B-chain of PDGF. These modifications fall basically into two classes: amino acid deletions and amino acid substitutions, including the cysteine and phenylalanine substitutions discussed below.

In regard to the deletion of amino acids, it has been found that the PDGF A-chain and B-chain may be truncated at either or both the amino- and carboxy-terminal ends and will still form biologically active molecules. Removal of these amino- and/or carboxy-terminal amino acids

results in smaller biologically active molecules, which may have broader therapeutic utility. Amino acids which may be deleted without destroying the biological activity of the resultant molecule include A-chain residues 1 through 22 and 96 through 104. Particularly preferred truncated A-chain analogs consist of amino acids 1 through 95, 9 through 95, 23 through 95, 9 through 104, and 23 through 104, although it will be evident to those skilled in the art that other polypeptides may also be constructed while still providing a molecule having biological activity. Further, amino acids which may be deleted without destroying the biological activity of the resultant B-chain molecule include residues 1 through 28 and residues 102 through 109. Particularly preferred truncated B-chain analogs consist of amino acids 1 through 101, 15 through 101, 29 through 101, 15 through 109, and 29 through 109, although it will be evident to those skilled in the art that other polypeptides may also be constructed while still providing a molecule having biological activity.

In addition, a variety of amino acid substitutions are possible. Preferred amino acid substitutions include replacement of selected cysteine residues with another amino acid, e.g., serine, as well as the replacement of other amino acids, the substitution of which does not destroy the biological activity of the resultant molecule. While the dimerization of the proteins of the present invention involves disulfide bonding between the component chains, it has been found that not all of the cysteine residues participate in the formation of disulfide bonds or are necessary for biological activity. Cysteine residues at positions 43, 54 and 91 of the A-chain are essential for the formation of biologically active molecules. Cys 93 may also contribute to proper structure. The cysteine at position 10 may not be required for the formation of biologically active molecules. The remaining cysteines at positions 37, 46, and 47 are not required for the formation of active dimers. Therefore, proteins having amino acid

substitutions at residues 10, 37, 46, 47 or 93 may also be suitable for use within the present invention, such as within a method for enhancing the wound-healing process in warm-blooded animals. Further, A-chain molecules containing more than one cysteine to serine mutation may also be biologically active. Preferred combinations include serine substitutions at positions 37 and 46 or at positions 37 and 47. Combinations of serines at positions 37 and 10 or 93 may also be suitable within the present invention.

Further, cysteine residues at positions 49, 60 and 97 of the B-chain are essential for the formation of active molecules. Cys 99 may also contribute to proper structure. The cysteine at position 16 may not be required for the formation of active dimers. The remaining cysteines at positions 43, 52 and 53 are not required for the formation of active molecules. Therefore, B-chain-like polypeptides having amino acid substitutions at residues 16, 49, 52, 53 or 99 may also be suitable for use within the present invention, such as within a method for enhancing the wound-healing process in warm-blooded animals. Further, B-chain molecules containing more than one cysteine to serine mutation may also be biologically active. Preferred combinations include serine substitutions at positions 43 and 52 or at positions 43 and 53. Combinations of serine substitutions at positions 43 and 16 or 99 may also be suitable within the present invention.

Other preferred amino acid substitutions include the replacement of a phenylalanine residue in the B-chain with a tyrosine residue. This substitution is useful, for instance, in facilitating the radioactive iodination of the B-chain as a laboratory reagent for use in binding and localization studies. The tyrosine residues are labelled with iodine under mild conditions which do not alter the biological activity of the protein. Preferred tyrosine for phenylalanine substitutions are at positions 23 and 37 of the B-chain. This was accomplished by oligonucleotide-directed mutagenesis following standard methodology. Oligo-

nucleotide ZC1116(5'-AGATCTCGTAAACTTCGG-3') was used to introduce the tyrosine substitution at position 23. Similar substitutions can be made for the other phenylalanine residues in the B-chain.

In addition to the amino acid substitutions described above, certain other amino acid substitutions are also possible, as long as the resultant polypeptide retains substantial homology to the A- or B-chain of PDGF. For example, a DNA sequence derived from the v-sis gene encodes a protein which, although homologous to the human B-chain, differs from the human amino acid sequence at four positions. Biologically active dimers may be made using the v-sis sequence or the authentic human sequence, which may be derived from a human cDNA or constructed by altering the v-sis sequence as described herein.

There are a variety of variants and derivatives which may be used within the present invention. For instance, amino acid substitutions may be made in either the A-chain or the B-chain which: (a) modify the three-dimensional structure of the particular chain without significantly effecting its biological activity; (b) modify the three-dimensional structure, resulting in an alteration of the biological activity; or (c) affect biological activity without significantly changing the three-dimensional structure. For example, amino acid number 98 in the B-chain may be changed from lysine to leucine, resulting in a monomer-sized molecule exhibiting biological activity. Alternatively, biologically active monomers may be obtained by changing cysteine residues involved in interchain disulfide bonds between the polypeptides of the dimer to an amino acid which will not form a disulfide bond. Molecules which are biologically active as monomers may permit greater therapeutic application. Monomer analogs can also be further manipulated without the requirement for the formation of a dimer to obtain biological activity. This will facilitate structural analysis, leading to the definition

of an active receptor binding site, thereby allowing the design of additional therapeutic analogs.

Further, deletion of one or more amino acids can also result in a modification of the structure of the resultant molecule without significantly altering its biological activity. This can lead to the development of a smaller active molecule which would have broader therapeutic utility. For example, as described herein, one can remove amino terminal amino acids not required for biological activity. Similarly, carboxy terminal amino acids may be removed, while retaining biological activity.

Further, mosaics of portions of the A- and B-chains or their derivatives may also be used within the present invention. The term "mosaic," as used within the present invention, includes contiguous portions of the A-chain and the B-chain sufficient to encode a molecule having biological activity as defined herein. The constituent portions of the mosaic can be chosen from wild-type A-chain or B-chain, as well as variants or derivatives of the A-chain or B-chain. The portions of the mosaic may range from 1 to approximately 75 amino acids in length, provided that the overall primary structural features of the A- and B-chains are maintained. The common structural features of the A- and B-chains are the relative positions of (a) the cysteine residues; (b) the regions of amino acid charge; and (c) regions of hydrophobic and hydrophilic character. In addition, it may be useful to maintain the blocks of sequence identity between the A- and B-chains.

As a further alternative, the sequences of these hybrids may be modified, while retaining biological activity. For example, one or more amino acid changes, such as a change at A-chain amino acid number 10 or in B-chain at amino acid 16 from a cysteine to a serine results in a molecule retaining biological activity.

Further, combinations of the A- and B-chains or their derivatives may also be used within the present invention. The term "combinations," as used within the present

invention, includes heterodimers composed of two different polypeptides; e.g., the A-chain and the B-chain. The constituent polypeptides of the heterodimer can be chosen from wild-type A-chain or B-chain or portions thereof, as well as variants or derivatives of the A-chain or B-chain. The DNA sequences to be utilized in expressing these polypeptides may be isolated, synthesized or constructed using standard recombinant DNA techniques.

In order to produce A-B heterodimers in yeast, constructs expressing both the A-chain and the B-chain of PDGF are introduced into the same yeast cell. In this way both polypeptide chains transit the secretory pathway simultaneously and are able to assemble into heterodimers. A preferred method is to place the A-chain and B-chain expression constructs on a single plasmid. In this way the copy number of the two sequences remains equal. It is also possible to introduce into and maintain within the same yeast cell separate plasmids, one encoding A-chain and the other B-chain.

Within the present invention, it has been found that by utilizing the secretory pathway of eucaryotic cells to express proteins substantially homologous or substantially identical to the A-chain or B-chain of PDGF or portions thereof, biologically active PDGF analogs may be obtained. Expression and secretion of these gene products from a eucaryotic cell enable processing and assembly, which result in molecules with native and biologically active conformation.

The secretory pathways of eucaryotes are believed to be quite similar. In particular, mammalian cell and yeast cell secretory pathways are well characterized and are homologous. The presence of a secretory signal sequence on the expressed polypeptide is an important element in eucaryotes, due to its role in directing the primary translation product into the secretory pathway, thereby leading to proper processing and assembly. Provided that appropriate transcriptional promoter and

secretory signal sequences are utilized, generally any eucaryote could express and secrete PDGF-analogs in a biologically active form.

An easily manipulable and well-characterized eucaryote is the yeast cell. For these reasons, yeast was chosen as a model example of an appropriate eucaryotic cell within the present invention. In accordance with the present invention, the yeast promoter is followed downstream by a DNA sequence which encodes a protein having substantially the same biological activity as PDGF. For example, DNA sequences encoding the 109 amino acids of the PDGF B-chain or the 104 amino acids of the A-chain were inserted into yeast extrachromosomal elements containing a yeast promoter capable of directing their expression. These extrachromosomal elements were transformed into yeast cells capable of expression and secretion of these biologically active PDGF analogs. In addition, variants and derivatives of the PDGF A- and B-chains, as well as the mosaic sequences, were also inserted into such a yeast extrachromosomal element.

The genes or sequences to be utilized in the present invention may be isolated using standard recombinant DNA techniques.

DNA sequences which encode a protein having substantially the same structure and/or biological activity as PDGF include the v-sis gene or derivatives of the v-sis gene, or portions thereof, or the human cDNAs for the A-chain or the B-chain of PDGF or portions thereof.

The human PDGF B-chain cDNA is isolated from a human cDNA library made from an appropriate source of messenger RNA, preferably by using the v-sis gene or a fragment thereof as a hybridization probe, or through use of oligonucleotide probes designed from the B-chain DNA sequence. A preferred source of mRNA is human umbilical vein endothelial cells. These cells can be cultured in vitro for short periods of time and are known to secrete PDGF into the culture medium (DiCorleto and Bowen-Pope, PNAS 80: 1919, 1983) and contain high levels of B-chain

mRNA. Breifly, polyadenylated RNA was prepared from freshly cultured human umbilical vein endothelial cells and used to make double-stranded cDNA by conventional techniques. This cDNA was cloned in bacteriophage lambda gt11. The resulting cDNA library was screened with probes derived from the v-sis gene. A second such library was made and screened in a similar fashion. Clones identified in this manner were mapped by restriction enzyme analysis in order to establish their extent of overlap. The identity of these clones as encoding PDGF B-chain was verified by DNA sequencing.

The human A-chain cDNA may be isolated from a human cDNA library made from an appropriate source of messenger RNA by using the v-sis gene or a fragment thereof as a hybridization probe, or through use of oligonucleotide probes designed from the A-chain DNA or amino acid sequence (see, for example, Betsholtz et al., Nature 320: 695-699, 1986). Preferred sources of mRNA are human transformed cell lines, e.g., U2-OS and T-24. These cells can be cultured in vitro and are known to secrete a protein having PDGF-like activity (Heldin et al., Nature 319: 511-514, 1986). The identity of this cDNA as that encoding A-chain may be verified by DNA sequencing.

In addition, suitable DNA sequences may be constructed using synthetic oligonucleotides.

Once an appropriate DNA sequence encoding a protein exhibiting PDGF-like biological activity is identified, the sequence is ligated to an appropriate promoter and secretory signal fragment. Promoters which may be utilized in yeast include the yeast alpha-factor (MFα1) promoter and the yeast triose phosphate isomerase (TPI1) promoter (Kawasaki, U.S. Patent No. 4,599,311). Promoters may also be obtained from other yeast genes, e.g., alcohol dehydrogenase 1 (ADH1), alcohol dehydrogenase 2 (ADH2). Appropriate promoters for other eucaryotic species may also be used and will be apparent to those skilled in the art. The constructions described herein were designed such that

the PDGF-related gene products would be secreted from the host cell into the media. In yeast, this was accomplished through use of the prepro secretory signal sequence of the yeast mating pheromone alpha-factor (Kurjan and Herskowitz, Cell 30: 933, 1982; Julius et al., Cell 36: 309, 1984; and Brake et al., PNAS 81: 4642, 1984), although other secretion signals may be used. To ensure the efficient transcription termination and polyadenylation of mRNA, a yeast terminator sequence, such as the TPI1 terminator (Alber and Kawasaki, J. Molec. Genet. Appl. 1: 419, 1982), was added. Methods of ligation of DNA fragments have been amply described (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory 1982) and are well within the skill of those of ordinary skill in the art to perform. After preparation of the expression unit constructions, the constructs are inserted into an appropriate expression vector.

It is preferable to use an expression vector which is stably maintained within the host cell in order to produce more biological activity per unit of culture. Suitable yeast expression vectors in this regard include the plasmids pCPOT and pMPOT2, which include the Schizosaccharomyces pombe gene encoding the glycolytic enzyme triose phosphate isomerase (POT1 gene). Inclusion of the POT1 gene ensures the stable maintenance of the plasmid in an appropriate host cell having a deletion in the TPI gene due to its ability to complement the host cell gene deletion. Other selection systems may also be used, such as the leu2 selection system described by Beggs (Nature 275: 104-109, 1978).

After preparation of the DNA construct incorporating the promoter, the alpha-factor secretory signal sequences, the appropriate DNA sequence encoding a molecule having PDGF-like biological activity, and the TPI terminator in an appropriate vector, the construct is transformed into the yeast host with a TPI deletion. Procedures for transforming yeast are well known in the literature (see,

for example, Beggs, ibid. and Hinnen et al., Proc. Natl. Acad. Sci. U.S.A. 75: 1929-1933, 1978).

The transformed yeast cells may be selected by growth on conventional complex medium containing glucose when the pCPOT or pMPOT2 vector is utilized. A conventional medium, such as YEPD (20 grams glucose, 20 grams Bacto-peptone, 10 grams yeast extract per liter), may be used. Once selected, transformants containing the appropriate expression constructions are grown to stationary phase on conventional complex media, the cells removed by centrifugation or filtration, and the medium concentrated. Noting that authentic human PDGF is a highly cationic and hydrophobic protein (Raines and Ross, ibid.; Antoniades, ibid.; Deuel et al., 1981, ibid.), it was expected that the yeast-expressed, PDGF-related products would possess similar characteristics, allowing the use of ion-exchange chromatography in their purification.

Using a variety of assays, it can be demonstrated that spent media from yeast cultures expressing the PDGF analogs possess biological activities substantially identical to authentic human PDGF.

Expression of biologically active PDGF analogs in eucaryotic cells other than yeast cells can be achieved by a person skilled in the art through use of appropriate expression/regulatory signals. Transcriptional promoters capable of directing the expression of these sequences are chosen for their ability to give efficient and/or regulated expression in the particular eucaryotic cell type. A variety of promoters are available, including viral (e.g., SV40 and adenovirus promoters) and cellular (e.g., metallothionein gene-karin, U.S. Patent No. 4,601,978) promoters. Signal sequences capable of directing the gene product into the cell's secretory pathway are chosen for their function in the appropriate cell type. Other useful regulatory signals, such as transcription termination signals, polyadenylation signals and transcriptional enhancer sequences, are also chosen for their function in the appropriate cell

type, the selection of which would be apparent to an individual skilled in the art. Methods for transforming mammalian cells and expressing cloned DNA sequences are described by Kaufman and Sharp (J. Mol. Biol. 159: 601-621, 1982), Southern and Berg (J. Mol. Appl. Genet. 1: 327-341, 1982), and Neumann et al. (EMBO J. 1: 841-845, 1982).

According to the present invention, it is possible to produce recombinant PDGF-like molecules which are homodimers or heterodimers of substantially identical polypeptide chains. To produce heterodimers, two different expression units are introduced into the same cell and heterodimers are identified among the biologically active products. The expression units may be on different expression vectors with different selectable markers or, preferably, on a single expression vector. The second strategy offers the advantage of providing equal copy numbers of the two expression units.

The techniques of cell culture have advanced considerably in the last several years as have the number and varieties of mammalian cells which will grow in culture. Central to these advances is a better understanding of the nutritional requirements (i.e., hormones and growth factors) of cultured cells (Barnes and Sato, Cell 22: 649, 1980). The types of cells able to grow in culture can be crudely classified in two groups: normal and transformed. So-called "normal" cells are generally not immortal in culture, they do not form tumors when injected into animals, and they retain a normal diploid karyotype. Normal cells may also retain much of their differentiated character in culture. Within the category of normal cells are those which will only grow for a limited number of generations in culture, termed "cell strains" or "primary cultures." Some normal cell lines, while not meeting all the criteria of transformation, may grow indefinitely in culture. Transformed cells are immortalized for growth in culture, typically have lost their differentiated phenotype, and have acquired karyotypic aberrations. They may also be

independent of anchorage for growth and induce tumors when injected into the appropriate host animal. Cells in any of these categories which grow in vitro and possess PDGF receptors will be responsive to the PDGF analogs of this invention.

As noted above, the proteins described herein are suitable for use within therapeutic compositions for enhancing the wound-healing process in warm-blooded animals. The normal wound-healing process in warm-blooded animals proceeds by an orderly series of events involving the interaction of chemoattractants, growth factors, and a variety of specialized cell types. This process includes an ordered migration and, in some cases, the subsequent proliferation of a number of these specialized cell types into the wound space, and involves the complex interaction of a variety of biologically active factors. This process is discussed in detail in Hunt et al., eds., Soft and Hard Tissue Repair: Biological and Clinical Aspects, Praeger Publishers, New York, 1984, which is hereby incorporated by reference. Briefly, tissue injury results in the release of chemotactic factors which attract particular cell types, which then release additional and/or other chemoattractant or mitogenic factors. These factors, in turn, affect additional specialized cells, ultimately restoring the injured tissue. Further, there is evidence that the rate at which this process normally proceeds is limited by the levels of chemoattractants and growth factors at the wound site, and may be enhanced by the addition of these agents (Grotendorst et al., J. Clin. Invest. 76: 2323-2329, 1985, herein incorporated by reference).

The wound-healing process in the dermis begins with the formation of a clot from the blood which flows into the wound. This results in a cross-linked network of fibrin molecules binding the wound together. During this process, platelets adhere to the injured tissue, becoming activated, and release the contents of their alpha granules. The disruption of the dermal tissue, the blood coagulation

reactions, and platelet activation all generate molecules which cause the migration of a series of new cells into the wound, thereby initiating the repair process.

Among the contents of the alpha granules released by the platelets is PDGF. In addition, other contents of the alpha granules and by-products of the coagulation reactions induce the appearance of macrophages. Macrophages are a second important source of PDGF in the wound. The deposition of PDGF at the site of an injury provides a chemotactic stimulus for fibroblasts to enter the wound space and a mitogenic stimulus for the fibroblasts to subsequently proliferate therein, thereby participating in the process of repair. An important role of the fibroblast is the regeneration of connective tissue at the wound site. The fibroblasts proliferate in the wound and deposit collagen types I and II and other extracellular proteins to the connective tissue matrix. The presence of new fibroblasts and their protein products reconstitutes the dermal architecture such that it can be re-epithelialized and the wound thereby healed.

Similarly, the wound-healing process in relation to the repair of connective tissue also requires fibroblast infiltration and proliferation, leading to subsequent collagen deposition.

The proteins of the present invention have been shown to possess substantially the same biological activity as authentic PDGF. The basic biological activity of PDGF, particularly the induction of chemotaxis and mitogenesis in responsive cell types (inlcuding fibroblasts and smooth muscle cells), underlies many of the physiological roles of this protein, including its role in tissue repair.

Because the chemotactic and mitogenic properties of PDGF are central to its role in the wound-healing process, the biologically active proteins of the present invention will have similar therapeutic utility. These biologically active proteins are therefore expected to have clinical applicability in the treatment of wounds in which

healing requires the migration and/or proliferation of fibroblasts. In addition, PDGF acts as a chemotactic and mitogenic agent for smooth muscle cells, the proliferation of which may contribute to the healing of certain wounds. Smooth muscle cells will be affected by PDGF in a manner similar to that described above for fibroblasts, thereby contributing to the healing process.

In individuals with normal healing capacity, exogenous proteins having the biological activity of PDGF accelerate the rate of appearance of fibroblasts in the wound and their subsequent proliferation. In addition, there are a large number of individuals who have substantially impaired wound healing capacity, and thereby lack the ability to provide to the wound site endogenous growth factors which are necessary for the process of wound healing. In these individuals, the addition of exogenous proteins having the biological activity of PDGF enables wound healing to proceed in a normal manner.

The proteins of the present invention are expected to accelerate the healing process in a broad spectrum of wound conditions. For purposes of the present invention, the terms "wound" or "wound condition" include any disruption of the dermal layer of the skin. Examples of disruptions to the dermal layer include chronic non-healing dermal ulcers (which can have a variety of causes), superficial wounds and lacerations, abrasions, surgical wounds, and some burns. In addition, wounds may also result in damage to connective tissue, the repair of which involves fibroblast proliferation and collagen deposition. The proteins of the present invention are useful in enhancing the healing process of all of these wounds, and will also be useful in the treatment of other wounds in which healing requires the migration and/or proliferation of fibroblasts. Furthermore, normal wound-healing may be retarded by a number of factors, including advanced age, diabetes, cancer, and treatment with anti-inflammatory drugs or anticoagulants; and the proteins described herein

may be used to offset the delayed wound-healing effects of such treatments. Lawrence et al., (Ann. Surgery 203: 142-147, 1986) demonstrated that PDGF restored the wound-healing process to normal in diabetic rats. Knighton et al., (Ann. Surgery 204: 322-330, 1986) used a mixed growth factor preparation comprising PDGF on chronic non-healing dermal wounds of human patients and observed dramatic positive results. Their results indicate that some of the activity in their preparation is due to PDGF and that PDGF contributes to the rapid healing they see in humans as it does in animal experiments. PDGF acts synergistically with other components of the preparation.

For therapeutic use in the applications described herein, the proteins of the present invention are preferably administered topically in combination with a physiologically acceptable carrier or diluent. Further, it is preferable to use a substantially pure preparation of the protein, that is, one which is generally free of impurities or contaminants which would interfere with its therapeutic use. Particularly preferred are those preparations which are free of toxic, antigenic, inflammatory or other deleterious substances, and are greater than 80% pure. Typically, the proteins desired herein will be in a concentration of about 1 to 50 µg/ml of total volume, although it will be apparent that concentrations in the range of 10 µg/ml to 100 µg/ml may be used. However, it should be noted that concentrations in excess of 50 µg/ml may result in reduced therapeutic effectiveness. A therapeutically effective amount sufficient to accelerate the rate of appearance and increase the number of new fibroblasts in the wound space and to stimulate DNA synthesis in and collagen deposition by those fibroblasts, will typically be in the range of 1 to 5 milliliters of the preparation, depending upon the characteristics of the wound.

Therapeutic compositions according to the present invention comprise the proteins described herein in combination with suitable carriers, as well as adjuvants, dilu-

ents, or stabilizers. Suitable adjuvants include collagen or hyaluronic acid preparations, fibronectin, factor XIII, or other proteins or substances designed to stabilize or otherwise enhance the active therapeutic ingredient(s). Diluents include albumins, saline, sterile water, etc. Other stabilizers, antioxidants, or protease inhibitors may also be added. Alternatively, the proteins may be applied to wound dressings as aqueous solutions. The therapeutic compositions according to the present invention may be reapplied at one- to several-day intervals until healing is complete.

The therapeutic compositions of the present invention may also contain other pharmaceutically active ingredients, for example, heparin, which has been shown to accelerate the healing of thermal burns. Other growth factors, such as TGF-α, TGF-β, EGF, FGF, platelet factor 4, insulin or somatomedins (see Grotendorst et al., 1985) and angiogenesis factor, may also work synergistically with the PDGF analogs described herein. Antibiotics may also be included to keep the wound free of infection.

To summarize the examples which follow, EXAMPLE I demonstrates the construction of a v-sis subclone of pSSV-11 in the E. coli replicating plasmid pUC13, subsequently designated pVSIS/Pst. EXAMPLE II demonstrates the construction of the plasmid pVSα, which includes the ligation of v-sis to the MFα1 promoter and secretory signal sequence. EXAMPLE III demonstrates the oligonucleotide-directed deletion mutagenesis of the first 195 base pairs of the v-sis gene using a technique which employs single stranded bacteriophage M13 in order to eliminate the first sixty-six amino acids of the v-sis gene product, $p28^{sis}$, which are not homologous to the B-chain of PDGF. A resulting phage with the correct deletion was designated m11vs2α. EXAMPLE IV demonstrates the construction of the expression vector pVSBm. EXAMPLE V demonstrates the transformation of yeast host cells. EXAMPLE VI demonstrates the construction of pSB1. EXAMPLE VII demonstrates the construction of vari-

ants and derivatives of the B-chain. EXAMPLE VIII demonstrates the construction of variants and derivatives of the A-chain. EXAMPLE IX demonstrates the construction of yeast expression vectors for A- and B-chain variants and derivatives. EXAMPLE X demonstrates a method for producing an A-B heterodimer in yeast. EXAMPLE XI demonstrates the concentration of the spent yeast growth media from transformed cultures and subsequent analysis for PDGF-like material. Clear evidence is presented that these yeast media containing the PDGF analogs described herein possess substantially the same biological activity as authentic human PDGF. EXAMPLE XII demonstrates methods for optimizing protein expression.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLE I

Subcloning of v-sis from pSSV-11

The SSV retroviral genome was cloned from SSV-11 nonproductively infected normal rat kidney (NRK) cells which had SSV integrated into their genome (Devare et al., 1982, ibid.). The SSV DNA was isolated as a 5.8 kilobase (kb) Eco RI fragment and subsequently inserted into the plasmid pBR322, resulting in the clone pSSV-11. This clone was obtained from S. Aaronson (National Institutes of Health, Bethesda, MD).

Figure 1A is a schematic restriction map of the 5.8 kilobase proviral genome of SSV. Only the restriction sites relevant to the present invention are indicated. The open box designates the $p28^{sis}$ coding portion of the v-sis gene.

Figure 1B depicts the nucleotide sequence of the v-sis gene and some flanking SSV sequences. The v-sis gene is inserted 19 nucleotides 3' of the putative ATG initiation codon of the envelope (env) gene of SSV (Devare

et al., 1982, ibid.). It is believed that transcription and translation of v-sis sequences are directed by SSV sequences resulting in an env-sis fusion protein. The nucleotide sequence shown in Figure 1B is corrected from that published by Devare et al. in 1982 (ibid.). The corrections include those made by Devare et al. in 1983 (ibid.) and by the inventors herein. The original numbering scheme of Devare et al. (1982, ibid.) is retained here for ease of reference. The numbers assigned to the restriction sites in Figure 1A are from Figure 1B.

A subclone of pSSV-11 (Figure 2) containing a portion of the v-sis gene was constructed in the E. coli replicating plasmid pUC13 (Vieira and Messing, Gene, 19: 259, 1982; and Messing, Meth. in Enzymology 101: 20, 1983). five micrograms (µg) of pSSV-11 was digested with the restriction endonuclease Pst I and the 1.2 kb fragment containing sequences numbered 454-1679 (Figure 1) was purified by agarose gel electrophoresis (0.9%) and extracted from the gel with cetyltrimethylammonium bromide (CTAB) plus butanol (Langridge et al., ibid.). Two µg of pUC13 was also digested with Pst I, phenol/chloroform ($CHCl_3$) extracted and ethanol (EtOH) precipitated. Forty ng of the 1.2 kb v-sis fragment and 50 ng of Pst I-cut pUC13 were ligated overnight at room temperature with 40 units (u) of T_4 DNA ligase. The ligation mixture was used to transform E. coli K-12 strain JM83 (Messing, Recombinant DNA Technical Bulletin, NIH Publication No. 79-009, 2, No. 2, 43-48, 1979) in the presence of 5-bromo, 4-chloro, 3-indolyl-ß-D-galactoside (X-gal) and isopropyl ß-D-thio-galactoside (IPTG). Plasmid DNA prepared from ampicillin-resistant white colonies was digested with Pst I to verify the presence of the insert and the resulting plasmid was designated pVSIS/Pst.

EXAMPLE II

Construction of the Plasmid pVSα

A. Preparation of v-sis for Fusion to MFα1.

Six hundred µg of plasmid pSSV-11 (Figure 2) was digested with restriction endonucleases Bam HI and Pvu II in 200 microliters (µl) of 50 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris pH 7.5 (medium salt buffer), and 100 µg/ml bovine serum albumin (BSA), overnight at 37°C. The digestion products were electrophoresed through a 1.1% agarose gel and the 1100 base pair (bp) Bam HI--Pvu II fragment (Figure 2) cut out, extracted and EtOH precipitated. The DNA pellet was dissolved in 75 µl Hph I buffer to which was added 20 µl of 1 mg/ml BSA and 5 µl Hph I. After overnight digestion at 37°C, the mixture was electrophoresed through a 1.25% agarose gel and the 396 bp Hph I--Pvu II fragment isolated from the gel and EtOH precipitated. The DNA pellet was dissolved in 30 µl of Klenow buffer (6mM Tris pH 7.5, 6 mM $MgCl_2$, 60 mM NaCl) and the 3' overhanging nucleotide at the Hph I cleavage site removed by treatment with 5 u of Klenow polymerase for 5 minutes at 37°C. One µl of a mixture containing all four deoxyribonucleotides each at 1 mM was added and the reaction mixture incubated an additional 10 minutes. After phenol/$CHCl_3$/ether (Et_2O) extraction and EtOH precipitation, the DNA pellet was dissolved in 30 µl of medium salt buffer and digested with 5 u of Bgl II for three hours at 37°C. The DNA was electrophoresed through a 1.25% agarose gel and the 269 bp Hph I--Bgl II fragment extracted and EtOH precipitated. The Hph I cleavage terminus of this Klenow blunted fragment begins with the tri-nucleotide sequence $\begin{matrix}5'\text{ATG}.....\\3'\text{TAC}.....\end{matrix}$ (Figure 2).

B. MFα1 Promoter and Secretory Leader Fragment.

Plasmid p192 (Figure 3) comprises a portion of the gene for the yeast mating pheromone α-factor (MFα1 gene) cloned in the bacterial plasmid pUC13 (Vieira and Messing, ibid.; and Messing, Meth. in Enzymology 101: 20, 1983). Cloning of the MFα1 gene from a genomic library has been described by Kurjan and Herskowitz (ibid.). The gene was isolated in this laboratory in a similar manner, using as starting material a yeast genomic library of partial Sau 3A fragments cloned into the Bam HI site of Yep13 (Nasmyth and Tatchell, Cell 19: 753, 1980). From this library, a plasmid was isolated which expressed α-factor in a diploid strain of yeast homozygous for the matα2-34 mutation (Manney et al., J. Cell Biol 96: 1592, 1983). The clone contained an insert overlapping with the MFα1 gene characterized by Kurjan and Herskowitz (ibid.). This plasmid, known as pZA2 (Figure 3), was cut with Eco RI and the 1700 bp fragment comprising the MFα1 gene was purified. This fragment was then subcloned into the Eco RI site of pUC13 to produce the plasmid p192.

Fifteen µg of plasmid p192 was digested in 30 µl of medium salt buffer with 20 units of Hind III overnight at 37°C. The reaction mixture was diluted to 60 µl with Klenow buffer and the four deoxyribonucleotides added to a final concentration of 50 µM each. Ten units of Klenow polymerase were added to the ice-cold mixture and incubation allowed to proceed 12 minutes at 15°C. Following phenol/$CHCl_3$/Et_2O extraction, the aqueous phase was concentrated by lyophilization to a volume of 10 µl and digested with 20 units of Eco RI for 70 minutes at 37°C. The products were electrophoresed through a 0.9% agarose gel and the 1.2 kb Eco RI--Hind III (blunted) MFα1 fragment extracted and EtOH precipitated. This DNA fragment contains the transcriptional promoter and secretory signal sequences of MFα1.

C. Preparation of v-sis 3' Sequences and Cloning Vector pUC12; Fragment Ligation.

Twenty µg of plasmid pVSIS/Pst was digested with Bgl II and Xba I in 40 µl of medium salt buffer. Subsequent electrophoresis through 1% agarose, extraction of the DNA and EtOH precipitation provided the purified v-sis 756 bp Bgl II--Xba I fragment (Figure 2). E. coli replicating plasmid pUC12 (5 µg) was digested with Eco RI and Xba I and gel-purified as above (Figure 2).

Referring to Figure 2, equimolar amounts of the four DNA fragments described above, adjusted to 10 ng of the 296 bp Hph I--Bgl II v-sis fragment, were mixed in 15 µl of ligase buffer (6 mM Tris pH 7.6, 6.6 mM $MgCl_2$, 0.4 mM ATP, 2 mM spermidine, 20 mM DTT, and 100 µg/ml BSA) and ligated with 40 units of T_4 DNA ligase overnight at 14°C. The reaction mixture was brought to room temperature, an additional 150 units of T_4 ligase added, and incubated 10 more hours. Seven µl of the ligation mix was used to transform E. coli K-12 RR1 (ATCC #31343; Bolivar et al., Gene 2: 95, 1977), and ampicillin-resistant transformants selected. Plasmid DNA was prepared from twelve such bacterial colonies and digested with Xba I. Two clones gave a 2.2 kb band predicted by the proper fragment alignment (Figure 2). Further analysis of these by Bgl II--Xba I restriction mapping gave expected bands of approximately 1.5 kb from the MFα1/v-sis fusion and 760 bp for the Bgl II--Xba I v-sis fragment. DNA sequence analysis verified the desired nucleotide sequence at the MFα1/v-sis junction. The resultant plasmid was designated pVSα.

EXAMPLE III

Construction of m11VS2α

Homology between the v-sis protein $p28^{sis}$ and PDGF begins at amino acid 67 of $p28^{sis}$, a serine residue corresponding to the NH_2 terminal residue of the PDGF B-chain (Johnsson, ibid.)

Proteolytic processing of the MFα1 primary translation product occurs at the Lys-Arg cleavage signal 85 amino acids from the initiator methionine (Kurjan and Herskowitz, ibid.). A v-sis derivative was constructed in which the first 66 codons of $p28^{sis}$ were removed such that serine residue 67 of v-sis immediately follows the MFα1 Lys-Arg processing signal.

Referring to Figure 4, approximately 40 ng of the gel purified 2.2 kb Xba I fragment of pVSα was ligated with 120 ng of Xba I digested, alkaline phosphatase-treated M13mp11 DNA (Messing, Meth. in Enzymology, ibid.). The ligation mixture was used to transform E. coli K-12 strain JM101 (ATCC 33876) in the presence of X-gal and IPTG. Isolated white plaques were picked and used to infect 3 ml cultures of log phase growth JM101 cells. Replicative Form (RF) DNA was prepared and clones identified which carried the insert fragment in the same orientation as the positive (+) strand form of the single-stranded mature phage. Single-stranded phage DNA was prepared from one such clone and designated m11VSα.

To precisely remove codons 1-66 of v-sis, oligonucleotide-directed mutagenesis was performed essentially according to the two-primer method of Zoller et al. (Manual for Advanced Techniques in Molecular Cloning Course, Cold Spring Harbor Laboratory, 1983). Oligonucleotide ZC 130 3' AGAAACCTATTTTCCTCGGACCCA 5' was synthesized on an Applied Biosystems 380-A DNA synthesizer. Fifty pmoles of ZC 130 was kinased in 10 µl of kinase buffer (BRL) with 4 units of

T_4 polynucleotide kinase for 45 minutes at 37°C. The enzyme was inactivated by heating at 65°C for 10 minutes.

One-half pmole of m11VSα was annealed with 1 pmole of kinased ZC130 and 1.5 pmoles of universal sequencing primer (BRL) using conditions described (Zoller et al., ibid.), except that the annealing mixture was first heated to 65°C for 10 minutes, shifted to 37°C for 10 minutes, and then quickly chilled on ice. The annealed mixture was then treated with Klenow polymerase as described by Zoller et al. (ibid.) to create circular duplex DNA. Portions of the elongation mixture were used to transform E. coli K12 JM101 cells. The resulting phage plaques were screened for the proper deletion by transfer onto nitrocellulose filters and subsequent hybridization with ^{32}P-phosphorylated ZC130 at 65°C. Correctly juxtaposed sequences formed stable duplexes with the radioactive probe at the stringent hybridization temperature employed. Approximately 1% of the transformants screened gave positive signals by autoradiography. Ten clones were plaque-purified and RF DNA was prepared for restriction enzyme analysis. Five isolates showed the expected decrease in size of 195 bp to the 1450 bp Hind III--Bgl II fragment (Figure 4). DNA sequence analysis of two isolates confirmed the correct fusion junction had been made, thus maintaining the proper translational reading frame. One of these phage was designated m11VS2α.

EXAMPLE IV

Construction of pVSBm

A. Construction of Plasmids YEpVSα and YEpVS2α.

Yeast-replicating vector YEp13 (Broach et al., Gene 8: 121, 1979) was used as an expression vehicle for v-sis-derived constructions described in Examples II and III. YEp13 is a multicopy extrachromosomal plasmid contain-

ing a 2 micron replication origin and the yeast LEU2 gene. This allows for selection of the plasmid in yeast strains possessing a defective chromosomal LEU2 gene when grown on synthetic medium lacking leucine. Addition of yeast terminator sequences to foreign genes expressed in yeast ensures efficient transcription termination and polyadenylation of mRNA. The v-sis expression units VSα and VS2α were placed adjacent to the TPI terminator fragment which was previously cloned into YEp13 (below).

Plasmid p270 (see Figure 5) contains the transcription terminator region of the yeast triose phosphate isomerase (TPI) gene. It was constructed in the following manner. The yeast TPI terminator fragment was obtained from plasmid pFG1 (Albert and Kawasaki, ibid.). It encompasses the region from the penultimate amino acid codon of the TPI gene to the Eco RI site approximately 700 base pairs downstream. A Bam HI site was substituted for this unique Eco RI site of pFG1 by first cutting the plasmid with Eco RI, then blunting the ends with DNA polymerase I (Klenow fragment), adding synthetic Bam HI linkers (CGGATCCA), and re-ligating to produce plasmid p136. The TPI terminator was then excised from p136 as a Xba I--Bam HI fragment. This fragment was ligated into YEp13 (Broach et al., ibid.), which had been linearized with Xba I and Bam HI. The resulting plasmid is known as p213. The Hind III site was then removed from the TPI terminator region of p213 by digesting the plasmid with Hind III, blunting the resultant termini with DNA polymerase I (Klenow fragment), and recircularizing the linear molecule using T_4 DNA ligase. The resulting plasmid is p270.

Alternatively, p270 may be constructed by digesting plasmid pM220 (see below) with Xba I and Bam HI, purifying the TPI terminator fragment (~700 bp) and inserting this fragment into Xba I and Bam HI digested YEp13.

Referring to Figure 6, plasmid p270 DNA was digested with Xba I and treated with calf alkaline phosphatase to prevent religation of the cohesive vector ends.

V-sis expression units VSα and VS2α were prepared by Xba I digestion and agarose gel purification of pVSα and m11vs2α, respectively. Each of the isolated fragments was ligated with an approximately equimolar amount of phosphatased p270 vector in the presence of 40 units of T_4 DNA ligase and the ligation mixtures transformed into E. coli K-12 RR1. Plasmid DNA was prepared from ampicillin-resistant colonies and restriction enzyme analysis performed in order to identify clones which possessed the TPI terminator adjacent to 3' v-sis sequences. Presence of 3.3 kb or 3.1 kb Bgl II fragments after gel electrophoresis indicated the correct orientation of YEpVSα and YEpVS2α, respectively.

B. Construction of the Plasmid pVSB.

Because the product encoded by pVS2α is larger than authentic human PDGF B-chain and because a smaller product might result in higher expression levels in a transformed yeast host cell, a vector was constructed comprising the v-sis sequence of pVS2α truncated at the 3' end. The polypeptide encoded by this sequence comprises amino acids 67 to 175 of $p28^{sis}$ and is homologous to the B-chain of PDGF.

An expression vector containing this "B-chain" sequence was constructed by combining elements of the pVS2α expression unit with a partial v-sis gene and a synthetic double-stranded DNA fragment encoding amino acids 158 to 175 of $p28^{sis}$. This synthetic fragment was designed to substitute preferred yeast codons for many of the 13 v-sis codons it replaces, and to supply a stop codon at the end of the coding sequence. The construction of this vector is illustrated in Figures 7 and 8.

Plasmid YEpVS2α was digested with Pst I and Bam HI; and the 1.8 kb fragment, comprising the partial MFα1, v-sis, and TPI terminator sequences, was purified by agarose gel electrophoresis. Plasmid pIC19R (Marsh et al., Gene 32: 481-486, 1984), comprising the polylinker shown in

Chart 1 inserted into the Hind III site of pUC19 (Norrander et al., Gene 26: 101-106, 1983), was digested with Pst I and Bam HI, and the vector fragment was gel-purified and joined to the 1.8 kb fragment from pVS2α to produce plasmid pVS2αT.

CHART 1

GAATTCATCGATATCTAGATCTCGAGCTCGCGAAAGCTT
Eco RI Eco RV Bgl II Sac I Hind III
Cla I Xba I Xho I Nru I

The S. cerevisiae TPI promoter was used to control expression of VS2α sequences in a yeast expression vector. Plasmid pM220 contains the TPI promoter fused to the MFα1 signal sequence. E. coli RRI transformed with pM220 has been deposited with American Type Culture Collection under accession number 39853.

Plasmid pM220 was digested with Bgl II and Pst I (Figure 7), and the ca. 1 kb fragment comprising the TPI promoter and the 5' portion of the MFα1 sequence was isolated and cloned in Bgl II + Pst I-digested pIC19R. The resultant plasmid was digested with Cla I and Pst I, and the TPI promoter--MFα1 fragment was gel-purified. Plasmid pVS2αT was then cut with Cla I and Pst I and joined to the TPI promoter--MFα1 fragment. The correct construct was identified by the presence of a 2.6 kb Cla I--Bam HI fragment and was designated pTVS2αT.

Ten µg of plasmid pVSα was digested with Xma I and Sph I (Figure 8) to completion. The resulting ca. 4.9 kb vector fragment, which also comprises most of the v-sis sequence, was purified by agarose gel electrophoresis, extraction of the DNA and EtOH precipitation.

In order to supply a new 3' terminus for the v-sis sequence, a double-stranded DNA fragment was constructed from oligonucleotides synthesized on an Applied Biosystems Model 380-A DNA synthesizer. 0.7 pmole of oligonucleotide

ZC299 (Table 1) was heated with an equimolar amount of oligonucleotide ZC300 in a volume of 10 µl containing 40 mM NaCl for 5 minutes at 65°C.

TABLE 1

ZC299: 5'TAAG TGT GAA ATC GTT GCC GCG GCT AGA GCT GTT ACC TAA TCT AGA3'

ZC300: 3'GTACA TTC ACA CTT TAG CAA CGG CGC CGA TCT CGA CAA TGG ATT AGA TCT GGCC5'

The mixture was then incubated at 37°C for 5 minutes and allowed to cool to room temperature. 0.2 pmole of the purified 4.9 kb vector fragment was added, the mixture ligated for 18 hours at 12°C and used to transform E. coli HB101 (ATCC 33694) to ampicillin resistance. DNA was prepared from ampicillin-resistant colonies and digested with Bgl II and Xba I. After electrophoresis through agarose, the desired clone (known as pVSαB) was identified by loss of a ca. 750 bp Bgl II--Xba I fragment and appearance of two smaller fragments of approximately 500 and 260 bp.

Approximately 8 µg of plasmid pTVS2αT (Figure 8) were digested to completion with Xba I in a volume of 10 µl. The volume was increased to 40 µl with Bgl II buffer, and 6 units of Bgl II were added and the mixture was incubated at 37°C. Ten µl aliquots were removed to a stop buffer containing 50 mM EDTA at 15 and 30 minutes, and the remaining 20 µl stopped at 45 minutes. The resulting mixtures were separated by electrophoresis through 0.7% agarose. The ca. 4.6 kb Bgl II--Xba I vector fragment was cut out, extracted from the gel, and EtOH precipitated. Plasmid pVSαB was digested with Bgl II and Xba I, and the ca. 260 bp fragment containing the synthetic 3' terminus and stop codon was isolated by electrophoresis through agarose, subsequent extraction from the gel, and EtOH precipitation.

The 4.6 kb Bgl II--Xba I vector fragment from pTVS2αT and the 260 bp Bgl II--Xba I fragment from pVSαB were ligated in the presence of T_4 DNA ligase for 7 hours at room temperature. The reaction mixture was used to transform E. coli HB101 to ampicillin resistance. DNA was prepared from transformants and the presence of the desired insert was confirmed by screening for a 550 bp Pst 1--Xba I band on an agarose gel. A plasmid having the correct configuration was designated pVSB.

There are several alternative approaches which can be used to construct plasmid pVSB. The essential elements of pVSB include: the TPI promoter/alpha-factor fusion, which can be obtained from plasmid pM220, the B-chain coding sequence (base 551 through 877 of Figure 1B) of the v-sis gene, which is widely available, and the TPI terminator, which can be obtained from plasmid p270. Someone skilled in the art could develop several strategies to arrive at pVSB using these elements.

C. Construction of pMPOT2.

In order to achieve maximal protein production from a yeast culture, it is desirable to use expression vehicles which are very stably maintained in the host cell. Plasmid pCPOT is such a preferred expression vehicle.

E. coli HB101 transformed with pCPOT has been deposited with American Type Culture Collection under accession number 39685. Plasmid pCPOT comprises the 2 micron circle genome (Hartley and Donelson, Nature 286: 860, 1980), E. coli plasmid pBR322 replication and selection sequences, and the Schizosaccharomyces pombe DNA sequences encoding the glycolytic enzyme triose phosphate isomerase (POT1). Presence of the POT1 gene in pCPOT ensures stable maintenance of the plasmid in the appropriate host background during growth on nonselective medium utilizing glucose as a carbon source.

For expression of the v-fos derivatives in yeast, a stable expression vector comprising the REP1, REP2, REP3 and ori sequences from yeast 2 micron DNA and the Schizosaccharomyces pombe triose phosphate isomerase (POT1) gene was constructed. The POT1 gene provides for plasmid maintenance in a transformed yeast host grown in complex media if such host is defective for triose phosphate isomerase.

The POT1 gene was obtained from the plasmid pFATPOT. S. cerevisiae strain E18 transformed with pFATPOT has been deposited with ATCC under accession number 20699. The plasmid may be purified from the host cells by conventional techniques. The POT1 sequence was removed from pFATPOT by digestion of the plasmid with Sal I and Bam HI. This ~1600 bp fragment was then ligated to pIC19R, which had first been linearized by digestion with Sal I and Bam HI. The Bam HI, Pst I and Sal I sites in the resultant plasmid were destroyed in two steps to produce plasmid pICPOT*. The Pst I and Sal I sites were removed by cutting with Pst I and Sal I; the ends were blunted by digesting the Pst I 3' overhang with DNA polymerase I (Klenow fragment) and filling in the Sal I 5' overhang with Klenow fragment. The blunt ends were then ligated. The Bam HI site was then removed by cutting the plasmid with Bam HI, filling in the ends with DNA polymerase I (Klenow fragment) and re-ligating the blunt ends.

The 2u sequences were obtained from the plasmids YEp13 (Broach et al., Gene 8: 121-133, 1979) and C1/1. C1/1 was constructed from pJDB248 (Beggs, Nature 275: 104-109, 1978) by removal of the pMB9 sequences by partial digestion with Eco RI and replacement by Eco RI-cut pBR322. The REP3 and ori sequences were removed from YEp13 by digestion with Pst I and Xba I and gel purification. REP2 was obtained from C1/1 by digestion with Xba I and Sph I and gel purification. The two fragments were then joined to pUC18 (Norrander et al., Gene 26: 101-106, 1983) which had been linearized with Pst I and Sph I to produce plasmid pUCREP2,3. REP1 was obtained from C1/1 by digestion with

Eco RI and Xba I and gel purification of the 1704 bp fragment. The Eco RI--Xba I fragment was cloned into pUC13 which had been linearized with Eco RI and Xba I. The resultant plasmid was designated pUC13 + REP1. The pUC13 + REP1 plasmid was cut with Hind II and ligated in the presence of Eco RI linkers (obtained from Bethesda Research Laboratories). The REP1 gene was then removed as an Eco RI fragment of approximately 1720 bp. This Eco RI fragment was cloned into pIC7 (Marsh et al., ibid.), which had been linearized with Eco RI and Xba I. The resultant plasmid was designated pICREP1#9.

To construct the final expression vector pMPOT2 (Figure 8), pICPOT* was linearized by a partial Hind III digestion and complete Sst I digestion. Plasmid pUCREP2,3 was cut with Hind III and Sst I, and the fragment comprising REP2, REP3 and ori sequences was gel-purified and joined to the linearized pICPOT*. The resultant plasmid, comprising REP2, REP3, ori, POT1 and amp^r sequences, was designated pMPOT1. REP1 was then removed from pICREP1 as a Bgl II--Nar I fragment and was ligated to pMPOT1, which had been cleaved with Bgl II and Nar I. The product of this ligation was designated pMPOT2 (deposited with ATCC, accession number 20744). Plasmid pMPOT2 was digested with Cla I and Bam HI, and the vector fragment was purified as above.

D. Insertion of VSB expression unit into pMPOT2.

Plasmid pVSB was digested with Cla I and Bam HI, and the 2.2 kb fragment containing the "B-chain" expression unit purified by agarose gel electrophoresis and EtOH precipitation. Plasmid pMPOT2 was also digested with Cla I and Bam HI. The fragments were ligated overnight at room temperature in the presence of T_4 DNA ligase and the reaction mixture used to transform E. coli HB101 to ampicillin resistance. DNA was prepared from transformants and the presence of the insert verified by digestion with Cla I and

Bam HI and agarose gel electrophoresis. The resulting expression vector was designated pVSBm (Figure 8).

EXAMPLE V

Yeast Transformation

Plasmids pVSBm and pMPOT2 were used to transform S. cerevisiae strain E18 #9 by conventional methods. Strain E18 #9 is a diploid produced by crossing strains E11-3c (ATCC No. 20727) (Δtpi::LEU2 pep4 leu2 MATα) and Δtpi29 (Δtpi::LEU2 pep4 leu2 his MATa). Δtpi29 is produced by disrupting the triose phosphate isomerase gene of strain E2-7b (ATCC No. 20689), essentially as described by Rothstein (Meth. in Enzymology 101: 202-210, 1983).

EXAMPLE VI

Construction of pSB1

In order to begin replacing B-chain coding sequence with A-chain sequence in the pVSB vector, a convenient Sst I restriction endonuclease site was created close to the α-factor prepro-B-chain boundary (Figure 8). This was accomplished by oligonucleotide-directed mutagenesis (Zoller and Smith, DNA 3: 479-488, 1984) on a single-stranded pVSB template using established techniques. The mutagenic oligonucleotide used is termed ZC506 and can be seen in Table 2.

TABLE 2

ZC505	GAACCCAGGCTTGCAGCTGGCAAAGATACCCC
ZC506	GGCTCCTTTTGAGCTCAGATACCCCT
ZC545	GATCTCGTAGATAACGGTACGCGTCTTACAAACAGCTCTCTTGAGCT
ZC546	CAAGAGAGCTGTTTGTAAGACGCGTACCGTTATCTACGA
ZC547	CAAGAGATCTATCGAAGAAGCGGTACCAGCCGTTTGTAAGACGCGTGA

ZC548	GATCTCACGCGTCTTACAAACGGCTGGTACCGCTTCTTCGATAGATCT CTTGAGCT
ZC671	CGCGTCTTAGAAACAGCTG
ZC672	GTACCAGCTGTTTCTAAGA
ZC675	CAAGTGTGAAACCGTTGCTGCTGCTAGACCAGTTACCTAAT
ZC676	CTAGATTAGGTAACTGGTCTAGCAGCAGCAACGGTTTCACACTTGCATG
ZC685	CAAGAGATCCTTGGGTTCTTTGACCATCGCTGAA
ZC686	AGCTGGTTCAGCGATGGTCAAAGAACCCAAGGATCTCTTGAGCT
ZC687	CCAGCTATGATCGCTGAATGTAAGACCAGAACCGAAGTTTTCGA
ZC688	GATCTCGAAAACTTCGGTTCTGGTCTTACATTCAGCGATCAT
ZC692	GATCCCAAGATCCCAAGTTGACCCAACCTCTGCCAACTTC
ZC693	TTGGCAGAGGTTGGGTCAACTTGGGATCTTGG
ZC746	TTGATTTGGCCACCATGTGTTGAAGTTAAGAGATGTACTGGGTGT
ZC747	CAGTACATCTCTTAACTTCAACACATGGTGGCCAAATCAAGAAG
ZC748	TGTCAAACCTCGAGTGTTAAGTGTCAACCATCCAGAGT
ZC749	GATGGTTGACACTTAACACTCGAGGTTTGACAACACC
ZC750	TCACCACAGATCCGTTAAGGTTGCCAAGGTTGAATACGTTAGAAAGAA GCCAA
ZC751	AGCTTTGGCTTCTTTCTAACGTATTCAACCTTGGCAACCTTAACGGAT CTGTGGTGAACTCTG
ZC752	AGCTTAAGGAAGTTCAAGTTAGATTGGAAGAACACTTGGAATGTGCAT GCGCTACCACCTCTTTGAACCCAGACTACAGAGAATAAT
ZC753	CTAGATTATTCTCTGTAGTCTGGGTTCAAAGAGGTGGTAGCGCATGCA CATTCCAAGTGTTCTTCCAATCTAACTTGAACTTCCTTA
ZC ABA-1	CGCTTGTGCTACCACCTCTTTGAACCCAGACTACAGAGAATAAT
ZC ABA-2	CTAGATTATTCTCTGTAGTCTGGGTTCAAAGAGGTGGTAGCACAAGCG CATG

A Pst I--Xba I fragment of pVSB (Figure 8) was subcloned into the M13 phage vector mp19. Single-stranded template DNA was prepared from E. coli JM107 cultures infected with this recombinant phage and used in the following mutagenesis reaction. Five µl of M13 template DNA (0.5 picomole) were combined with 2 ml of oligonucleotide ZC506 (1.8 pmole) plus 2.5 µl of water and 1.5 µl of 10X annealing buffer A (0.2 M Tris-HCl, 0.0 M $MgCl_2$, 0.01 M DTT pH 7.5; Zoller and Smith, DNA 3: 479-488, 1984). This

mixture was annealed by heating to 70°C for 5 minutes, cooled slowly to room temperature and then placed on ice. To this cold annealing mixture was added 1.5 µl of 10X elongation buffer B (0.2 M Tris-HCl, 0.1 M $MgCl_2$, 0.1 M DTT pH 7.5, Zoller and Smith, ibid.), 6 µl of deoxynucleotide triphosphates (2.5 mM each dNTP), 1 µl of T_4 DNA ligase, 1 µl of DNA polymerase Klenow fragment, 1 µl ATP (10 mM), and 5 µl of water. This mixture was incubated for 16 hours at 18°C. This reaction mixture was then diluted 20-fold with water, and 2 µl of the dilute mixture was used to transform E. coli JM107 cells. The resulting phage plaques were transferred to nitrocellulose discs by the procedure of Benton and Davis (Science 196: 180, 1977) and screened with ^{32}P-labeled ZC506 which was labeled with T_4 polynucleotide kinase under standard conditions. The hybridization of the ^{32}P-ZC506 to the filters was performed at 37°C in 6X SSC (0.9 M NaCl, 0.09 M Na Citrate, pH 7.2), 100 µg/ml carrier DNA, 0.05% sodium pyrophosphate. Following hybridization, the filters were washed at 54°C in 6X SSC, 0.1% SDS. Phage plaques giving strong autoradiographic signals were picked and RF DNA made and analyzed for the presence of a new Sst I restriction endonuclease site. The sequence around the Sst I site was also confirmed by DNA sequence analysis. The Pst I-Xba I subclone now containing an Sst I site was ligated back into Pst I-Xba I digested pVSB and the resulting plasmid termed pSB1. Plasmid pSB1 encodes two amino acid changes (Leu to Glu and Asp to Leu) in the alpha-factor leader just upstream of the Lys-Arg. The resulting junction sequence is: α-factor . . . Glu Leu Lys Arg Ser . . . B-chain. The B-chain coding sequences of pSB1 are thus flanked by an Sst I site at the 5' end and an Xba site at the 3' end.

EXAMPLE VII

Construction of Variants and Derivatives of the B-chain

A. Construction of a Human B-chain Expression Unit.

The B-chain construction pVSB described in Example IV above encodes the monkey B-chain amino acid sequence derived from the v-sis gene. This B-chain amino acid sequence differs from the human B-chain amino acid sequence at four positions: 6, 7, 101 and 107. These amino acid differences are largely conservative and not likely to affect the biological activity of the B-chain.

In order to express authentic human B-chain, the monkey-specific amino acids were changed to the human sequence by incorporating synthetic oligonucleotide duplexes, encoding the human amino acids into the pVSB construction. In this case, the preferred starting vector was pSB1 (described above), which has an Sst I site introduced at the α-factor-B-chain junction. The DNA sequences between this Sst I site and the Bgl II site at amino acid #24 (Figure 9) were replaced to encode the human amino acids threonine and isoleucine at positions 6 and 7. Four oligonucleotides, ZC685, ZC686, ZC687 and ZC688 (Table 2), were designed to replace the pSB1 sequences between Sst I and Bgl II. ZC685 and ZC686 were annealed to form one duplex, and ZC687 and ZC688 were annealed to form the other. These two annealed duplexes were then ligated with Sst I-Bgl II digested pSB1 vector. The resulting plasmid was confirmed by DNA sequencing and termed pSB11.

The amino acid changes at the B-chain carboxyl end were made in a similar fashion. Plasmid pSB11 is digested with Sph I and Xba I and the sequences in this region were replaced by a synthetic DNA duplex designed to encode human amino acids threonine at position 101 and proline at position 107. Oligonucleotides ZC675 and ZC676 were annealed and ligated into Sph I-Xba I digested pSB11

under standard conditions. The construction was confirmed by DNA sequencing and termed pB12. This plasmid encodes the authentic human B-chain amino acid sequence.

Alternatively, the human B-chain amino acid sequence could be expressed by performing site-specific mutagenesis on the pVSB plasmid to change the four amino acids in question or by expressing a human B-chain cDNA sequence.

B. Monomer-Size B-chain Mutant.

Biologically active PDGF, as it is isolated from platelets or transformed cells in culture, is a disulfide bonded dimer. Chemical reduction of this dimer molecule destroys its biological activity. Surprisingly, it has been found that changing B-chain cysteine residues which are involved in interchain disulfide bonds to other amino acids or changing amino acids near these cysteine residues allows the B-chain polypeptide to fold properly but not permit interchain disulphide bonds to occur. This results in a monomer B-chain folded in a confirmation which permits binding to the PDGF cell surface receptor. Changing B-chain amino acid lysine 98 to a leucine has resulted in a molecule which is active as a monomer. This molecule is made as follows.

Plasmid pVSB (Figure 8) is digested with Sph I and Xba I, and the DNA sequences between these restriction sites are replaced with a synthetic oligonucleotide duplex. The duplex is formed by annealing oligonucleotides analogous to ZC299 and ZC300, but containing a leucine codon at position 98 instead of a lysine codon. All the other codons in this region are preserved and encode B-chain amino acid sequence. The annealed duplex has a 5' Sph I cohesive end and a 3' Xba I cohesive end and is ligated into the Sph I-Xba I digested pVSB. This B-chain mutant is termed pSB6.

When this construction is cloned into the pMPOT2 plasmid (then termed pSB6m) and transformed into yeast, it produces mitogenically active material. Furthermore, when the expressed mitogenic material is fractionated on a polyacrylamide gel and subsequently eluted from slices of the gel, mitogenically active material is found in the monomer size range of the gel. This demonstrates that it is possible to produce a biologically active PDGF monomer by altering cysteine residues or the environment around them. Mutagenesis of other cysteine residues in the molecule may therefore be expected to lead to a similar result.

C. Truncated Amino Terminal B-chain Mutant.

During biosynthesis of the B-chain in the yeast expression system, the α-factor prepro polypeptide is removed from the B-chain by proteolytic processing at the basic dipeptide, Lys-Arg. Another basic dipeptide Arg-Arg occurs 27 amino acids downstream in the B-chain (Figure 9). It was of interest to know if the yeast processing machinery would process the B-chain at this internal site and still yield an active protein. In order to drive the proteolytic processing to occur at the internal Arg-Arg site, the Lys-Arg at the α-factor-B-chain boundary was removed by oligonucleotide directed mutagenesis.

The mutagenesis was performed essentially as described for the construction of pSB1 above. The Pst I--Xba I fragment of pVSB (Figure 8) was subcloned into the M13 phage vector mp19 and single-stranded template DNA prepared. In this case, the mutagenic oligonucleotide used, ZC505 (Table 2) , was designed to change the α-factor Lys-Arg residues to Gly-Leu and to introduce a new Pvu II restriction site. The mutagenesis reactions were carried out as described above for pSB1 and the resulting mutants screened for the new Pvu II site and then confirmed by DNA sequence analysis. The mutagenized Pst I-Xba I fragment

was subcloned back into the B-chain expression unit (pVSB) and the new plasmid termed pSB3.

EXAMPLE VIII

Construction of Variants and Derivatives of the A-chain

A. Synthesis of the A-chain Amino Terminus and Construction of A-B Hybrid Fusions.

The A-chain coding sequences were inserted into the pSB1 vector as short synthetic oligonucleotide duplexes designed to encode known A-chain amino acid sequence (Johnson et al., EMBO J. 3: 921-928, 1984). ZC545 and ZC546 (Table 2) were annealed, creating a short duplex DNA fragment with a 5' Sst I cohesive end, a unique Mlu I restriction site, and a 3' Bgl II cohesive end. This duplex was cloned into Sst I and Bgl II-digested pSB1. One ul of pSB1 vector (0.15 pmole) was combined with 1 µl of ZC546 (~1.6 pmole) and 0.6 µl of ZC545 (~1.5 pmole), plus 0.25 µl of 0.3 M NaCl (final NaCl concentration in the annealing reaction is 30 mM) and the mixture was heated to 60°C for five minutes. After heating, the mixture was brought to room temperature and then placed on ice. Then 0.5 µl of 10X ligase buffer (0.5 M Tris-HCl, 0.1 M $MgCl_2$, 2 M DTT, 0.01 M ATP, pH 7.8), 0.1 µl of T_4 DNA ligase (New England Biolabs) and 2.5 µl of water were added and this ligation mixture was diluted and used to transform E. coli HB101 cells. Ampicillin-resistant, plasmid-bearing colonies were picked, grown up and plasmid DNA isolated by the "miniprep" method of Ish-Horowicz and Burke (Nuc. Acid Res. 9: 2989-2998, 1981). The plasmids were analyzed for the presence of an Sst I-Bgl II insert and a new Mlu I restriction site and confirmed by DNA sequence analysis. The ZC545-546 duplex encoded A-chain amino acids alanine 8 through tryosine 17 (Figure 9) and the resulting plasmid was termed pA1.

ZC547 and ZC548 (Table 2) were annealed to create a second short Sst I--Bgl II fragment encoding A-chain amino acids serine 1 through arginine 13 (Figure 9) and also containing an Mlu I restriction site. The ZC547-548 duplex was separately cloned into Sst I and Bgl II digested pSB1. One µl of pSB1 (1.5 pmole) digested with Sst I and Bgl II was combined with 2 µl of ZC547 (1 pmole) and 2 µl of ZC548 (1 pmole) plus 0.25 µl of 0.3 M NaCl and the mixture was heated to 50°C for five minutes. After heating, this annealing mixture was brought to room temperature and then placed on ice. Then 0.6 µl of 10X ligase buffer and 0.1 µl of T_4 DNA ligase (New England Biolabs) were added and the reaction was incubated overnight at 12°C. An aliquot of this ligation reaction was diluted and used to transform E. coli HB101 cells and the resulting plasmids were screened and analyzed as described above for pA1. In this case, the resulting plasmid was termed pA2.

The overlapping pA1 and pA2 A-chain coding regions were joined at the unique Mlu I restriction site using conventional techniques. Plasmid pA2 was digested with Mlu I and Bam HI and the ~1.4 kb vector (pUC containing) fragment was isolated by agarose gel electrophoresis and extracted from the agarose with CTAB (Langridge et al., Anal. Biochem. 103: 264-271, 1980). Plasmid pA1 was also digested with Mlu I and Bam HI and the ~800 base pair fragment, encoding A-chain amino acids 13 through 17 fused to B-chain amino acids 24 through 109 followed by the TPI terminator, was isolated and extracted as above. Equimolar amounts of these two fragments were ligated under standard conditions and an aliquot used to transform E. coli HB101 cells. Plasmids obtained from ampicillin-resistant colonies were analyzed by restriction enzyme digestion for the correct fragments and confirmed by DNA sequencing. The resulting plasmid termed pA3 thus encoded a hybrid protein beginning with A-chain amino acids 1 through 17 followed in frame by B-chain amino acids 24 through 109. The Cla I--Bam HI fragment of pA3 containing the entire expression

unit was cloned into pMPOT2 and the resulting plasmid pA3m was transformed into yeast.

Further addition of A-chain amino acids to the A-B hybrid was accomplished in a similar fashion. Plasmid pA3 was digested first with Asp718, which cuts the plasmid once in the A-chain sequence at proline codon 7, and with Bam HI, and the hybrid amino acid coding fragment subcloned into pUC118. This subclone was termed pA3N and was subsequently digested with Bgl II and Bst XI. Bgl II cuts at the boundary of the A- and B-chain sequences in the hybrid and Bst XI cuts approximately 40 base pairs downstream in the B-chain. The vector fragment (pUC-containing) from this digest was isolated by agarose gel electrophoresis and extracted with CTAB. One picomole each of oligonucleotides ZC692 and ZC693 (Table 2) was annealed to form a short DNA duplex with a 5' Bgl II end and a 3' Bst XI end. This duplex encoded A-chain glutamic acid 18 through phenylanine 31 and was ligated with 0.1 picomole of Bgl II-Bst XI-digested pA3N. The ligation was performed overnight and the ligated products transformed into E. coli MV1193 cells. The resulting plasmid termed pA6N now has extended the A-chain amino acid sequence to the Bst XI site at amino acid A31 followed by B-chain amino acids B38 through B109.

Plasmid pA6N was then digested with Asp718 and Bam HI and the A-B hybrid fragment cloned back into Asp718-Bam HI digested pA3m. This new A-B hybrid plasmid is termed pA6m and encodes A-chain amino acid sequence up to amino acid 40 because the Bst XI site lies at the start of a region of high homology between A- and B-chains.

B. Construction of A-B-A Hybrid Fusions.

Since the A- and B-chains of PDGF are so homologous in structure and function, there are likely to be several biologically active hybrid molecules which can be made between the two. A number of examples containing amino terminal A-chain sequence followed by B-chain amino

acids are described above. Another example of this concept would be to construct a hybrid protein which contained A-chain amino acid sequence at the amino and carboxyl termini and B-chain sequence in the middle.

A preferred embodiment would use plasmid pA6, which encodes the hybrid protein A1-17, B24-109, and to exchange A-chain for B-chain amino acid sequence at its carboxyl end. In this case, the B-chain coding region is digested with the restriction endonuclease Sph I, which cuts in B-chain codons 96 and 97 (Figure 9). The plasmid pA6 is cut again with Xba I, which cuts immediately 3' of the translation termination codon. This sequence is then replaced with two oligonucleotides which, when annealed, form a duplex with 5' and 3' Sph I and Xba I cohesive ends, respectively, and contain A-chain codons (Figure 9). The two oligonucleotides, ZC ABA-1 and ZC ABA-2, are shown in Table 2. These two oligonucleotides are annealed by heating to 65°C and slow cooling as described above. The annealed duplex is then ligated into Sph I-Xba I digested pA6 plasmid which has been isolated by agarose gel electrophoresis. The ligated product is transformed into E. coli MV1193 cells and transformed colonies obtained on ampicillin plates. In this case, no new restriction sites are introduced by the new A-chain duplex, so the construction is confirmed by DNA sequence analysis.

C. Construction of an A-chain Cysteine Mutant.

As can be seen from Figure 9, both the A- and B-chains of PDGF contain eight cysteine residues which are capable of forming disulphide bonds. It can also be seen from Figure 9 that these cysteine residues are in analogous positions in the two polypeptides and hence may participate in similar disulfide arrangements in and between the two chains and even between two different chains (A and B). It has been known for several years that chemical reduction of the disulfide-bonded PDGF dimer to monomers destroys its

biological activity. It is of interest to know which of the cysteine residues in question are involved in disulfide bonds of both the intra- and intermolecular type. It is very likely that the role of each cysteine will be analogous in both A- and B-chains.

The first cysteine residue in the A-chain occurs at position #10, which is analogous to #16 in the B-chain (Figure 9). The A-B hybrid pA3 (described above) encodes A-chain amino acids 1-17, followed by B-chain. The synthetic strategy leading to construction of pA3 incorporated unique restriction sites flanking the cysteine at residue A10. An Asp718 and an Mlu I restriction site were placed 5' and 3', respectively, to the A10 cysteine codon approximately 20 base pairs apart. Two oligonucleotides (ZC671 and ZC672, Table 2) were synthesized and annealed to form a short DNA duplex with a 5' Asp718 cohesive end and a 3' Mlu I cohesive end. This duplex encodes a serine residue in place of cysteine A10. Plasmid pA3 was digested with Asp718 and Mlu I and the large vector (pUC containing) fragment isolated by agarose gel electrophoresis. Equimolar amounts of the vector and the ZC671-672 duplex were ligated under standard conditions as described above and then transformed into E. coli MV1193 cells. Plasmid (miniprep) DNA was prepared from the resulting transformants and screened for a new Pvu II site present in the ZC671-672 duplex. The duplex region of the plasmid is then confirmed by DNA sequence analysis. The resulting plasmid, termed pA5, encodes an A-B hybrid protein with A-chain amino acids 1-17 at the amino terminus, but residue 10 is a serine instead of a cysteine. The remaining amino acids of the pA5 hybrid are the normal B-chain residues (Glu 24 through Thr 109).

D. Complete Synthesis of the A-chain Gene.

The remainder of the A-chain gene was synthesized with oligonucleotides in a fashion very similar to that described above. Many strategies could be designed to

accomplish this task. One such strategy is described below. The oligonucleotides used in this strategy are shown in Table 2 and their design reflects optimal codon usage for Saccharomyces cerevisiae. In this strategy, the remainder of the A-chain gene was synthesized with unique restriction sites introduced in order to facilitate subcloning and sequencing the synthetic oligonucleotide sequences. All the oligonucleotides were synthesized on an Applied Biosystems 380-A DNA synthesizer. Oligonucleotides ZC752 and ZC753, each 87mers, were annealed and subcloned as a Hind III--Xba I fragment encoding A-chain amino acids 77-104. ZC752 and ZC753 (1.25 picomole each) were annealed in 5 µl of 40 mM NaCl by heating to 65°C for 15 minutes and then allowing the mixture to come to room temperature and putting on ice. One-tenth of this annealed duplex (.0125 picomole) was ligated into both pUC118 (.07 pmole) and M13 mp18 (.02 picomole) which were previously digested with Hind III and Xba I. The ligated mixtures were used to transform the appropriate E. coli host strain (JM107 in the case of M13 mp18 and MV1193 in the case of pUC118) and the resulting plasmid or RF DNAs analyzed by restriction endonuclease digestion and DNA sequencing.

The oligonucleotides ZC746 + 747, 748 + 749, and 750 + 751 were designed to form short duplexes with cohesive ends which when joined would constitute the sequence between the Bst XI site at A31 and the Hind III site at A77. The oligonucleotides were phosphorylated with ^{32}P and T_4 polynucleotide kinase under standard conditions. The pairs ZC746 + ZC747, ZC748 + ZC749, and ZC750 + ZC751 were each annealed by combining 2.5 pmole of each oligonucleotide in 5 µl of 40 mM NaCl, heating to 65°C for 15 minutes, allowing to come to room temperature, and putting on ice. The three annealing mixtures were combined (now 15 µl) and ligated in a final volume of 20 µl. The ligated products were electrophoresed in a 4% NuSieve agarose gel (FMC Corporation) in TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM disodium EDTA) followed by autoradiography. The 140

base pair fragment corresponding to the three correctly ligated duplexes was cut out of the gel and extracted with CTAB. This fragment, together with the previously cloned Hind III-Xba I fragment, was ligated into the Bst XI-Xba I digested pA6N vector. The resulting plasmid was termed pA6N+. Plasmid pA6N+ was then digested with Asp718 and Xba 1 and the A-chain coding fragment cloned back into pA3. This plasmid pA7 encodes the entire mature A-chain.

For purposes of yeast expression, a preferred embodiment would employ oligonucleotides ZC748 and ZC749. These encode a glutamine at position A-48 instead of an asparagine. This change destroys the N-linked glycosylation site which can be aberrantly glycosylated in yeast. Oligonucleotides designed to preserve the N-linked glycosylation site could also be used.

The strategy employing total gene synthesis described above is desirable because the amino acid sequence of the A-chain is known and the codon usage can be optimized for yeast. Alternatively, an A-chain cDNA sequence could be expressed in yeast or other eukaryotic cells, provided the cDNA was appropriately incorporated into a suitable expression vector. An A-chain cDNA could be obtained from a variety of mammalian cell lines by conventional techniques (Betsholtz et al., Nature 320, 695-699, 1986.)

E. Construction of A-chain Amino Terminal Truncated Mutant

During biosynthesis of the A-chain protein in the yeast expression system, the α-factor prepro-peptide is removed from the A-chain by proteolytic processing at the basic dipeptide Lys-Arg, alpha factor amino acid residues #84 and #85. In order to drive the proteolytic processing to occur at an internal A-chain site, the Lys-Arg at the α-factor-A-chain boundary is removed and an internal Arg-Arg created by oligonucleotide directed mutagenesis.

The Lys-Arg removal mutagenesis is performed essentially as described for the construction of pSB1 above. The Pst I--Xba I fragment of pVSB (Figure 8) is subcloned into the M13 phage vector mp19 and single-stranded template DNA is prepared. In this case, the mutagenic oligonucleotide is designed to change the α-factor Lys-Arg residues to Gly-Leu and to introduce a new Pvu II restriction site. The mutagenesis reactions are carried out as described above for pSB1 and the resulting mutants are screened for the new Pvu II site and then confirmed by DNA sequence analysis. The mutagenized Pst I--Xba I fragment is subcloned back into the A-chain expression unit (designated pA7).

In order to introduce a dibasic peptide site into the A-chain coding sequence, oligonucleotide-directed mutagenesis is employed as described above. Amino acid residue #22 in the A-chain is a serine, while #21 is an Arg. In this case, the mutagenic oligonucleotide is designed to change the Ser #22 to an Arg, creating the sequence Arg-Arg at positions #21 and #22. This new dibasic site in the A-chain occurs in a position precisely analogous to one which is normally present in the B-chain (Figure 9). By expressing this mutant construction from the α-factor leader lacking the dibasic processing site, the resultant A-chain molecule should be processed internally at the new Arg-Arg and be secreted as a truncated polypeptide.

EXAMPLE IX

Insertion of Expression Unit Constructions into pMPOT2

Each of the molecules constructed in Examples VI-VIII above was introduced back into the basic expression unit pVSB or pSB1 if the Sst I site was employed. Then each of them was ultimately cloned into the yeast plasmid pMPOT2 (Example IV). In each case, this was done by removing the expression unit as a single fragment from pVSB or

pSB1 by Cla I-Bam HI digestion. The Cla I--Bam HI fragment of each was isolated by agarose gel electrophoresis and cloned into pMPOT2 which had been digested with Cla I and Bam HI. The names of the resulting plasmids are then amended with a lower case "m," e.g., pA2 becomes pA2m.

Each of the mPOT constructions was then transformed into the yeast strain E18-#9 (Example VI).

EXAMPLE X

A-B Heterodimer Expression Construction

PDGF is a disulfide-bonded dimer, and as isolated from platelets, is composed of two amino acid chains, an A-chain and a B-chain. It is presumed that this material is in the form of an A-B heterodimer. There are now a number of examples of PDGF homodimers composed of either A- or B-chain (Kelly et al., EMBO J. 4: 3399-3405 1985; Stroobant and Waterfield , EMBO J. 3: 2963, 1984; and Heldin et al., Nature 319: 511, 1986). While platelet PDGF is still thought to be a heterodimer, these data raise the possibility that it may actually be a mixture of A-A and B-B homodimers.

Having expressed biologically active A and B forms of PDGF in the yeast expression system, it is possible to introduce both an A- and a B-chain expression unit into the same yeast cell and to identify A-B heterodimers among the biologically active products. This can be done by introducing the respective expression units into the yeast cell on different plasmids, possibly with different selectable markers. Using this strategy, it may be difficult to control the relative copy numbers of the two plasmids and this may result in disproportionate amounts of the A- and B-chain polypeptides in the cell. A preferred strategy would be to put both the A-chain and B-chain expression units on the same plasmid. In this way, their copy numbers would always be equal.

There are numerous ways to incorporate both expression units into the same plasmid. Within the present invention, the B-chain expression unit from plasmid pVSB (Figure 8) was removed by complete digestion with Bam HI followed by partial digestion with Bgl II. The fragment corresponding to the expression unit (TPI promoter-MFα1-B-chain-terminator) was isolated by agarose gel electrophoresis and subcloned into the Bam HI site of pUC18. The orientation of the insert in the resulting subclones was established by conventional restriction enzyme digestions. A subclone in which the Bgl II end of the insert was adjacent to the Sal I site in the polylinker was chosen for the next step. This subclone was digested with Sal I and Bam HI and the insert fragment isolated. This B-chain fragment was then ligated into plasmid pA7m, which had been digested with Sal I and Bam HI. The resulting plasmid pAB2m contains both the A- and the B-chain expression units oriented tail to tail. This plasmid is then transformed into yeast strain E18-#9.

EXAMPLE XI

Biological Activity Assays

A. Radioreceptor Assay (RRA) for PDGF.

The radioreceptor assay for PDGF (Bowen-Pope and Ross, J. Biol. Chem. 257: 5161, 1982) is a specific and sensitive (0.2-2 ng/ml PDGF) method for detecting biologically active PDGF-like material in yeast. In this assay, PDGF-like material is tested for its ability to compete with purified, radio-labeled ^{125}I-PDGF for binding sites on cell surface PDGF receptors. Results are interpreted by comparison to a standard curve generated with purified, unlabeled PDGF. Comparison of results obtained with other assay methods (e.g., ELISA) provides an indication of the strength of the receptor/ligand interaction in addition to

quantitation of the material bound. The assay is conducted as follows: Subconfluent monolayers of diploid human fibroblasts are prepared by plating 1.5 x 10^4 cells per 2 cm^2 culture well in Costar 24-well cluster trays in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 1% human plasma-derived serum (PDS). Cultures are set on an ice tray and rinsed once with ice-cold binding rinse (Ham's medium F-12 buffered at pH 7.4 with 25 mM HEPES and supplemented with 0.25% BSA). One ml/well of test substance in binding medium is added and the cultures incubated in a refrigerated room on an oscillating platform for 3 to 4 hours. The trays are then placed on ice, aspirated, rinsed once with cold binding rinse and incubated for one hour as above with 1 ml/well binding medium containing 0.5 ng/ml ^{125}I-PDGF. Labeling is terminated with four rinses of binding rinse and cell-associated ^{125}I-PDGF determined by extraction with solubilization buffer. Standard curves are obtained using 0, 0.05, 0.1, 0.2, 0.4, and 0.8 ng/ml purified PDGF and test samples compared to these values.

PDGF receptor binding by CM-Sephadex media concentrates from yeast transformants containing plasmids pVSBm and pMPOT2 was compared to receptor binding by authentic PDGF. After concentration by binding to and elution from CM-Sephadex, the pVSBm concentrate was normalized to PDGF equivalents in an ELISA using polyclonal goat antibody to PDGF. The RRA results were interpreted by comparison to a standard curve generated with purified, unlabeled PDGF, as shown in Figure 10. Media from cultures transformed with the pVSBm constructions are shown to compete with ^{125}I-PDGF for binding to the PDGF receptor. Media from yeast cells transformed with pMPOT2 do not compete with radio-labeled PDGF for receptor binding.

B. Mitogenesis Assay.

The ability of PDGF to stimulate DNA synthesis and cell growth in culture was the basis for its definition

and discovery. ^{3}H-Thymidine incorporation into DNA of cultured cells responsive to PDGF (Raines and Ross, Meth. in Enzomology 109: in press) is a preferred method for demonstrating the biological activity of PDGF-like molecules produced in yeast.

Straight spent media test samples or concentrates of spent media or test samples in 10 mM acetic acid (up to 100 µl/well) are added to quiescent cultures of mouse 3T3 cells in 2cm^2 Costar 24-well culture dishes (2-3 x 10^8 cells/well in 1 ml). Quiescent test cultures can be obtained by plating the cells in 10% serum and allowing them to deplete the medium, 4 to 5 days. The test samples are removed from the wells at 20 hours and replaced with 0.5 ml of fresh medium per well containing 2 uCi/ml [^{3}H]-Thymidine and 5% (v/v) calf serum. After an additional 2-hour incubation at 37°C the cells are harvested by: aspirating off the medium; washing the wells twice each with 1 ml of ice-cold 5% TCA; solubilizing TCA-insoluble material in 0.8 ml 0.25N NaOH with mixing; and counting 0.6 ml of this solution in 5 ml Aquasol in a liquid scintillation counter. Fold stimulation over control wells (100 µl of 10 mM acetic acid alone) is determined (normally 30- to 50-fold maximal stimulation) and compared to a standard curve obtained using purified PDGF preparations.

The A-chain homodimer and the B-chain homodimer have been purified to homogeneity, quantitated by amino acid analysis, and found to have substantially equal specific activity in the mitogenesis assay.

EXAMPLE XII

Optimization of Protein Expression

A. Optimized B-Chain Expression Construction

The DNA sequences encoding the alpha-factor leader and PDGF B-chain were modified to contain yeast-optimal codons and to encode wild-type alpha-factor as well as authentic human B-chain. This restored the alpha-factor sequence which had been altered in previous constructions and allowed the optimization of B-chain expression levels.

The codon-optimized alpha-factor leader sequence was obtained from an expression vector containing the gene for the insulin analog B(1-29)-Ala-Ala-Lys-A(1-21) (Markussen et al., EP 163,529). An Eco RI-Xba I fragment comprising the alpha-factor pre-pro and insulin sequences was cloned into Eco RI, Xba I digested pUC118 (obtained from J. Vieria and J. Messing, Waksman Institute of Microbiology, Rutgers, Piscataway, N.J.) and single-stranded template DNA was prepared. This template was then mutagenized according to the two-primer method (Zoller and Smith, DNA 3: 479-488, 1984) using the mutagenic oligonucleotide ZC862 (5' CGA ATC TTT TGA GCT CAG AAA CAC C 3'). The mutagenesis resulted in the creation of an Sst I site at the 3' end of the alpha-factor leader. A correctly altered plasmid was selected and designated pKP23. The leader sequence was excised from pKP23 by digestion with Eco RI and Sst I, and the leader fragment was subcloned into pIC19 (Marsh et al., Gene 32: 481-486, 1984). The resultant plasmid was designated pKP24.

The human B-chain sequence was obtained from plasmid pB12 (Example VII A). pB12 was digested with Sst I and Xba I and the B-chain fragment was recovered. Plasmid pKP10, comprising the TPI promoter--alpha-factor--VSB--TPI terminator of pSB1 (Example VI) inserted into a pBR322 vector lacking an Eco RI site, was digested with Sst I and Xba I to remove the VSB sequence. The pB12 B-chain sequence and the pKP24 alpha-factor sequence (Eco RI-Sst I) were then inserted into the pKP10 expression unit. The resultant plasmid was designated pKP26.

The Sst I site introduced into the alpha-factor leader to facilitate the construction of pKP26 was then removed to restore the wild-type coding sequence. Plasmid pKP26 was digested with Eco RI and Xba I and the alpha-factor--B-chain fusion sequence was recovered. This fragment was cloned into pUC118 and single-stranded template DNA was isolated. The template was mutagenized by the two primer method using the mutagenic oligonucleotide ZC1019 (5' ACC CAA GGA TCT CTT GTC CAA AGA AAC ACC TTC TTC 3'). A correctly mutagenized plasmid was designated pKP32.

The entire expression unit was then reconstructed. Plasmid pKP32 was digested with Eco RI and Xba I and the alpha-factor--B-chain fragment was recovered. This fragment was inserted into Eco RI, Xba I cut pKP25 to construct pKP34. Plasmid pKP34 was digested with Cla I and Bam HI and the expression unit was recovered. This fragment was inserted into Cla I, Bam HI digested pMPOT2 to construct pKP36.

The B-chain sequence was then codon optimized. An internal Bgl II--Sph I fragment of the B-chain sequence of pKP36 was replaced with a sequence assembled from the oligonucleotides shown in Table 4. The pMPOT2-based expression vector containing the fully optimized expression unit was designated pB170m.

B. Optimized A-Chain Expression Construction

The codon-optimized A-chain sequence from plasmid pA7 (Example VIII D) was combined with the codon-optimized alpha-factor leader sequence in a series of construction steps parallel to those described above for B-chain. The alpha-factor sequence of pKP24 was combined with the pA7 A-chain sequence and Eco RI, Xba I cut pKP10 to construct pKP27. Plasmid pKP27 was digested with Eco RI and Xba I and the alpha-factor--A-chain fragment was cloned into pUC118.

Mutagenesis, using the oligonucleotide ZC1018 (5' TTC GAT AGA TCT CTT GTC CAA AGA AAC ACC TCC TTC 3'), was carried out as described above to remove the Sst I site and restore the wild-type alpha-factor sequence. The corrected plasmid was designated pKP31.

A codon-optimized expression vector was then constructed. Plasmid pKP31 was digested with Eco RI and Xba I and the alpha-factor--A-chain fragment was joined to Eco RI, Xba I cut pKP24. The resultant vector, designated pKP33, contained the entire expression unit. Plasmid pKP33 was digested with Cla I and Bam HI and the expression unit fragment was recovered. This fragment was inserted into Cla I, Bam HI cut pMPOT2 to construct the expression vector pKP35.

C. Expression of A-Chain and B-Chain

S. cerevisiae strain XB13-5B was separately transformed with plasmids pB170m and pKP35 according to standard procedures. Transformants were cultured in glucose media at 30°C with agitation. Cultures were harvested, cells were removed by centrifugation, and protein was purified from the supernatants as described below.

S. cerevisiae strain XB13-5B transformants containing plasmids pB170m and pKP35 have been deposited

with American Type Culture Collection, Rockville, Md. 20852, U.S.A.

D. Protein Purification

Yeast culture supernatants (12 liters), prepared as described above, were concentrated on an Amicon RA2000 ultrafiltration membrane at a flow rate of 60 ml/min to a volume of 350 ml. The buffer was exchanged with 25 mM Na acetate pH 5.5 containing 0.1% NaN_3. The concentrated samples were centrifuged at 15,000 rpm for 20 min and stored at -20°C.

The frozen samples were thawed, centrifuged at 15,000 rpm for 20 min and fractionated on an S-Sepharose column (Pharmacia). 300 ml samples were loaded onto a 30 ml column at a flow rate of 2 ml/min. The column was then washed with 25 mM Na acetate pH 5.5 containing 0.1% NaN_3. The column was eluted at a rate of 2 ml/min using the following elution program: 20 min with 20 mM Na phosphate pH 7.3 containing 0.1% NaN_3; 30 min with 20 mM Na phosphate pH 7.3, 0.2 M NaCl; 50 min with 20 nM Na phosphate pH 7.3, 0.5 M NaCl; 20 min with 20 mM Na phosphate pH 7.3, 1M NaCl. A- and B-chain polypeptides eluted at 0.5 M NaCl. Fractions were assayed for mitogenic activity and by gel electrophoresis. Mitogenically active fractions were pooled and the pH adjusted to 6.0.

Final purification was accomplished by high-performance liquid chromatography (HPLC). The pooled fractions from the Sepharose chromatography were passed over a MicroPak C-18 column at a flow rate of 1 ml/min. The PDGF polypeptides were eluted using a gradient of Buffer B (0.1% TFA in CH3CN) in Buffer A (0.1% TFA in H20). The elution program was:

Time (min.)	% Buffer B
Ø	Ø
17.5	35
32.5	5Ø
33.Ø	1ØØ

0.5 ml fractions were collected and assayed for mitogenic activity, and active fractions were pooled.

TABLE 4

ZC886	GGCCACCATGTGTTGAAGTTCAAAGATGCTCGGGTTGTTGTAACAACAGAAAC GTTCAATG
ZC887	TCGACATTGAACGTTTCTGTTGTTACAACAACCCGAGCATCTTTGAACTTCAA CACATG
ZC888	GATCTCTAGAAGATTGATCGACAGAACCAACGCCAACTTCTTGGTTT
ZC889	GTGGCCAAACCAAGAAGTTGGCGTTGGTTCTGTCGATCAATCTTCTAGA
ZC907	CGTTAGAAAGAAGCCAATCTTCAAGAAGGCTACCGTTACCCTCGAGGACCACT TGGCATG
ZC908	TCGACCAACCCAAGTTCAATTGCGGCCGGTTCAAGTGCGCAAGATCGAAAT
ZC909	CTAACGATTTCGATCTTGCGCACTTGAACCGGCCGCAATTGAACTTGGGTTGG
ZC910	CCAAGTGGTCCTCGAGGGTAACGGTAGCCTTCTTGAAGATTGGCTTCTTT

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

The features disclosed in the foregoing description, in the claims and/or in the accompanying drawings may, both separately and in any combination thereof, be material for realising the invention in diverse forms thereof.

Claims

1. A DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells, said DNA construct containing a transcriptional promoter followed downstream by a DNA sequence encoding a polypeptide which is substantially identical to the A-chain of PDGF.

2. A DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells, said DNA construct containing a transcriptional promoter followed downstream by a DNA sequence, a portion of said DNA sequence encoding a polypeptide which is substantially identical to at least a portion of the A-chain of PDGF, and a portion of said DNA sequence encoding a polypeptide which is substantially identical to at least a portion of the B-chain of PDGF, said portions of said DNA sequence encoding a protein having substantially the same biological activity as PDGF.

3. The DNA construct of claim 2 wherein said DNA sequence encodes a polypeptide substantially identical to A-chain amino acids 1-17 fused in reading frame to B-chain amino acids 24-109.

4. The DNA construct of claim 2 wherein said DNA sequence encodes a polypeptide substantially identical to A-chain amino acids 1-17 fused in reading frame to B-chain amino acids 24-97 fused in reading frame A-chain amino acids 92-104.

5. A DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells, said DNA construct containing a transcrip-

tional promoter followed downstream by a DNA sequence encoding a biologically active protein monomer which is substantially homologous to the B-chain of PDGF.

6. A DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells, said DNA construct containing a transcriptional promoter followed downstream by a DNA sequence encoding a polypeptide chain substantially homologous to the A-chain of PDGF, and a transcriptional promoter followed downstream by a DNA sequence encoding a polypeptide chain substantially homologous to the B-chain of PDGF, said chains forming a heterodimer.

7. A DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells, said DNA construct containing a transcriptional promoter followed downstream by a secretory signal sequence lacking a proteolytic processing site, thereby resulting in proteolytic processing within the B-chain of PDGF, said signal sequence being followed downstream by a DNA sequence encoding a protein which is substantially homologous to the B-chain of PDGF.

8. The DNA construct of claims 1-7 wherein said polypeptide includes at least one amino acid substitution of a cysteine residue.

9. A DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells, said DNA construct containing a transcriptional promoter followed downstream by a DNA sequence encoding a polypeptide which is substantially homologous to the B-chain of PDGF, said polypeptide including at least one amino acid substitution of a cysteine residue.

10. A DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in

eucaryotic cells, said DNA construct containing a transcriptional promoter followed downstream by a DNA sequence encoding a polypeptide which is substantially homologous to a portion of the B-chain of PDGF from amino acid 29 to amino acid 109.

11. A method of preparing biologically active PDGF analogs, comprising:

introducing into a eucaryotic host cell a DNA construct according to any of claims 1-10;

growing said eucaryotic host cell in an appropriate medium; and

isolating the PDGF analog from said eucaryotic host.

12. A eucaryotic host cell transformed with a DNA construct according to any of claims 1-10.

13. A eucaryotic host cell transformed with a first DNA construct comprising a DNA sequence encoding a polypeptide which is substantially identical to the A-chain of PDGF, and a second DNA construct comprising a DNA sequence encoding a polypeptide which is substantially identical to the B-chain of PDGF.

14. A recombinant protein having two polypeptide chains, each of said chains being substantially identical to the A-chain of PDGF, said protein having substantially the same biological activity as PDGF.

15. A protein having two polypeptide chains, one of said chains being a mosaic of amino acid sequences substantially identical to portions of the A- or B-chains of PDGF, the second of said chains being substantially homologous to the A-chain of PDGF, said protein having substantially the same biological activity as PDGF.

16. A protein having two polypeptide chains, one of said chains being a mosaic of amino acid sequences substan-

tially identical to portions of the A- or B-chains of PDGF, the second of said chains being substantially homologous to the B-chain of PDGF, said protein having substantially the same biological activity as PDGF.

17. A protein having two polypeptide chains, each of said chains being a mosaic of amino acid sequences substantially identical to portions of the A- or B-chains of PDGF, said protein having substantially the same biological activity as PDGF.

18. The protein of claims 14-17 wherein said protein includes at least one amino acid substitution of a cysteine residue.

19. A protein having two polypeptide chains, each of said chains being substantially identical to the B-chain of PDGF, said protein including at least one amino acid substitution of a cysteine residue.

20. A protein monomer which is substantially homologous to the B-chain of PDGF, said protein having substantially the same biological activity as PDGF.

21. The protein of claims 14-20 wherein said protein is unglycosylated.

22. The protein of claim 14 wherein said polypeptide chains are substantially identical to (a) the A-chain of PDGF from amino acid 9 to amino acid 104; (b) the A-chain of PDGF from amino acid 23 to amino acid 104; (c) the A-chain of PDGF from amino acid 9 to amino acid 95; (d) the A-chain of PDGF from amino acid 23 to amino acid 95; or (e) the A-chain of PDGF from amino acid 1 to amino acid 95; or (f) the amino acid sequence of Figure 9, from A-chain amino acid 1 to amino acid 104.

23. The protein of claim 15 wherein said second polypeptide chain is substantially identical to (a) the A-chain of PDGF from amino acid 9 to amino acid 104; (b) the A-chain of PDGF from amino acid 23 to amino acid 104; (c) the A-chain of PDGF from amino acid 9 to amino acid 95; (d) the A-chain of PDGF from amino acid 23 to amino acid 95; or (e) the A-chain of PDGF from amino acid 1 to amino acid 95.

24. The protein of claim 15 or 16 wherein said mosaic chain is substantially identical to (a) A-chain amino acids 1-17 fused in reading frame to B-chain amino acids 24-109; or (b) A-chain amino acids 1-17 fused in reading frame to B-chain amino acids 24-97 fused in reading frame to A-chain amino acids 92-104.

25. The protein of claim 15 or 16 wherein said polypeptide chains are substantially identical to one another.

26. The protein of claim 16 wherein said second chain is substantially identical to (a) the B-chain of PDGF from amino acid 15 to amino acid 109; (b) the B-chain of PDGF from amino acid 29 to amino acid 109; (c) the B-chain of PDGF from amino acid 15 to amino acid 101; (d) the B-chain of PDGF from acid 29 to amino acid 101; (e) the B-chain of PDGF from amino acid 1 to amino acid 101; or (f) the amino acid sequence of Figure 9, from B-chain amino acid 1 to amino acid 109.

27. A composition comprising a protein according to any of claims 14-26, for use as an active therapeutic substance.

28. A therapeutic composition comprising a protein according to any of claims 14-26, and a physiologically acceptable carrier or diluent.

29. The therapeutic composition of claim 28 wherein said carrier or diluent is selected from the group consisting of albumin, sterile water, and saline.

30. The therapeutic composition of claim 28 or 29, including an adjuvant.

31. The therapeutic composition of claim 30 wherein said adjuvant is selected from the group consisting of collagen, hyaluronic acid, fibronectin, factor XIII, and an antibiotic.

32. The therapeutic composition of claim 28 wherein said protein is present in a concentration of from about 1 to 50 µg/ml of total volume.

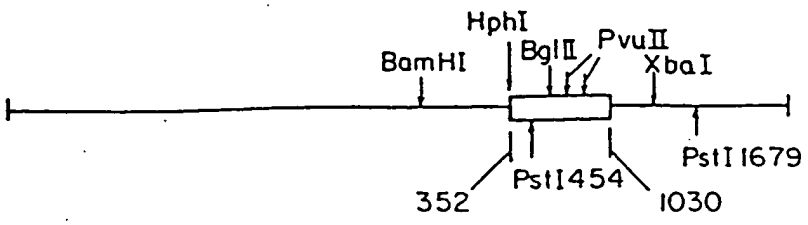
33. A composition according to any of claims 28-32, for use in enhancing the wound-healing process in warm-blooded animals.

34. A method of promoting the growth of mammalian cells, comprising incubating the cells with a protein according to any of claims 14-26.

35. A wound dressing containing a protein according to any of claims 14-26.

36. A recombinant protein having two polypeptide chains, each of said chains being substantially identical to the B-chain of PDGF, at least one of said chains having at least one amino acid substitution of a tyrosine residue for a phenylalanine residue.

FIG. 1A

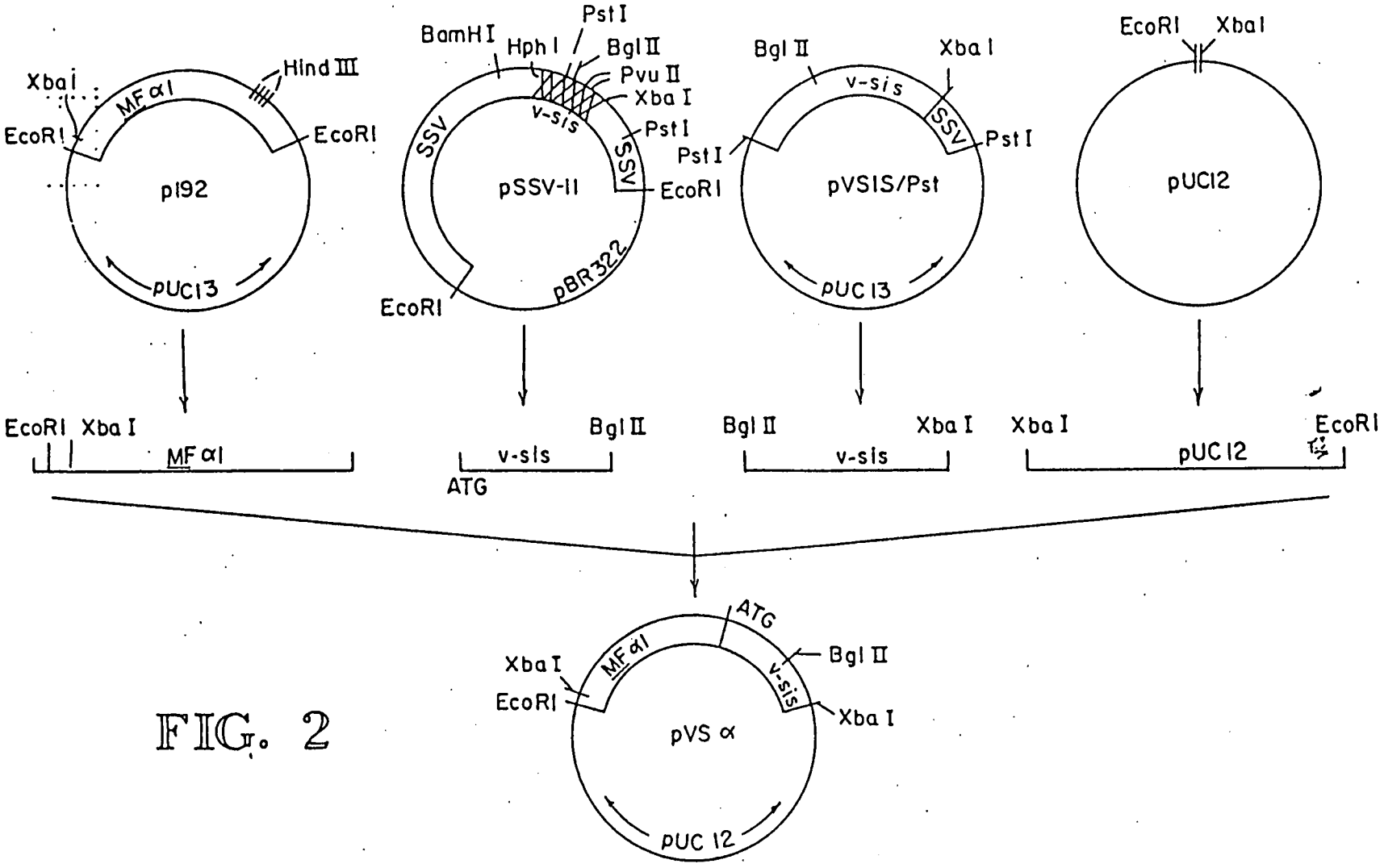



```
Hph I                          v-sis-helper viral junction
                          367                  382                  397
CT ATG ACC CTC ACC TGG CAG GGG GAC CCC ATT CCT GAG GAG CTC TAT AAG ATG
   MET Thr Leu Thr Trp Gln Gly Asp Pro Ile Pro Glu Glu Leu Tyr Lys MET

                                                                  Pst I
        412                  427                  442                  457
CTG AGT GGC CAC TCG ATT CGC TCC TTC AAT GAC CTC CAG CGC CTG CTG CAG GGA
Leu Ser Gly His Ser Ile Arg Ser Phe Asn Asp Leu Gln Arg Leu Leu Gln Gly

                  472                  487                  502
GAG TCC GGA AAA GAA GAT GGG GCT GAG CTG GAC CTG AAC ATG ACC CGC TCC CAT
Asp Ser Gly Lys Glu Asp Gly Ala Glu Leu Asp Leu Asn MET Thr Arg Ser His

    517                  532                  547                  562
TCT GGT GGC GAG CTG GAG AGC TTG GCT CGT GGG AAA AGG AGC CTG GGT TCC CTG
Ser Gly Gly Glu Leu Glu Ser Leu Ala Arg Gly Lys Arg Ser Leu Gly Ser Leu

            577                  592                  607
AGC GTT GCC GAG CCA GCC ATG ATT GCC GAG TGC AAG ACA CGA ACC GAG GTG TTC
Ser Val Ala Glu Pro Ala MET Ile Ala Glu Cys Lys Thr Arg Thr Glu Val Phe

  Bgl II
622                  637                  652                  667
GAG ATC TCC CGG CGC CTC ATC GAC CGC ACC AAT GCC AAC TTC CTG GTG TGG CCG
Glu Ile Ser Arg Arg Leu Ile Asp Arg Thr Asn Ala Asn Phe Leu Val Trp Pro
```

FIG. 1B

```
        682                   697                   712                   727
CCC TGC GTG GAG GTG CAG CGC TGC TCC GGC TGT TGC AAC AAC CGC AAC GTG CAG
Pro Cys Val Glu Val Gln Arg Cys Ser Gly Cys Cys Asn Asn Arg Asn Val Gln

                          | Pvu II
                742                   757                   772
TGC CGG CCC ACC CAA GTG CAG CTG CGG CCA GTC CAG GTG AGA AAG ATC GAG ATT
Cys Arg Pro Thr Gln Val Gln Leu Arg Pro Val Gln Val Arg Lys Ile Glu Ile

    787                   802                   817                   832
GTG CGG AAG AAG CCA ATC TTT AAG AAG GCC ACG GTG ACG CTG GAG GAC CAC CTG
Val Arg Lys Lys Pro Ile Phe Lys Lys Ala Thr Val Thr Leu Glu Asp His Leu

                          Pvu II
            847           862           877
GCA TGC AAG TGT GAG ATA GTG GCA GCT GCA CGG GCT GTG ACC CGA AGC CCG GGG
Ala Cys Lys Cys Glu Ile Val Ala Ala Ala Arg Ala Val Thr Arg Ser Pro Gly

892                   907                   922                   937
ACT TCC CAG GAG CAG CGA GCC AAA ACG ACC CAA AGT CGG GTG ACC ATC CGG ACG
Thr Ser Gln Glu Gln Arg Ala Lys Thr Thr Gln Ser Arg Val Thr Ile Arg Thr

        952                   967                   982                   997
GTG CGA GTC CGC CGG CCC CCC AAG GGC AAG CAC CGG AAA TGC AAG CAC ACG CAT
Val Arg Val Arg Arg Pro Pro Lys Gly Lys His Arg Lys Cys Lys His Thr His

                1012                  1027          1043        1053
GAC AAG ACG GCA CTG AAG GAG ACC CTC GGA GCC TAA GGGCATCGGC AGGAGAATAT
Asp Lys Thr Ala Leu Lys Glu Thr Leu Gly Ala

      1063        1073        1083        1093        1103        1113        1123
GGGCAGCGGG TCTCCTGCCA GCGGCCTCCA GCATCTTGCC CAGCAGCTCA AGAAGAGAAA AAAGGACTGA

      1133        1143        1153        1163        1173        1183        1193
ACTCCACCAC CATCTTCTTC CCTTAACTCC AAAAACTTGA AATAAGAGTG TGAAAGAGAC TGATAGGGTC

      1203        1213        1223        1233        1243        1253        1263
GCTGTTTGAA AAAAACTGGC TCCTTCCTCT GCACCTGGCC TGGGCCACAC CCAAGTGCTG TGGACTGGCC

      1273        1283        1293        1303        1313        1323        1333
CGAGGGGCCC TGCACGTGGC CCTGAGCACC TCTCAGTGTA GCCTGCCTGG TCCCTAGACC CCTGGCCAGC

                                                  XbaI | v-sis-helper viral junction

      1343        1353        1363        1373
TCCAAGGGGA GGCACCTCCA GGCAGGCCAG GCTACCTCGG GGGTCTAG
```

FIG 1D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Pst I
EcoR I
SSV
v-sis
SSV
pSSV-11
pBR322
EcoR I
Bgl II
v-sis
Xba I
Pst I
SSV
Pst I
pVSIS/Pst
pUC 13
EcoR I
Xba I
pUC12
EcoR I Xba I
MF α1
Bgl II
v-sis
ATG
Bgl II
Xba I
v-sis
Xba I
EcoR I
pUC 12
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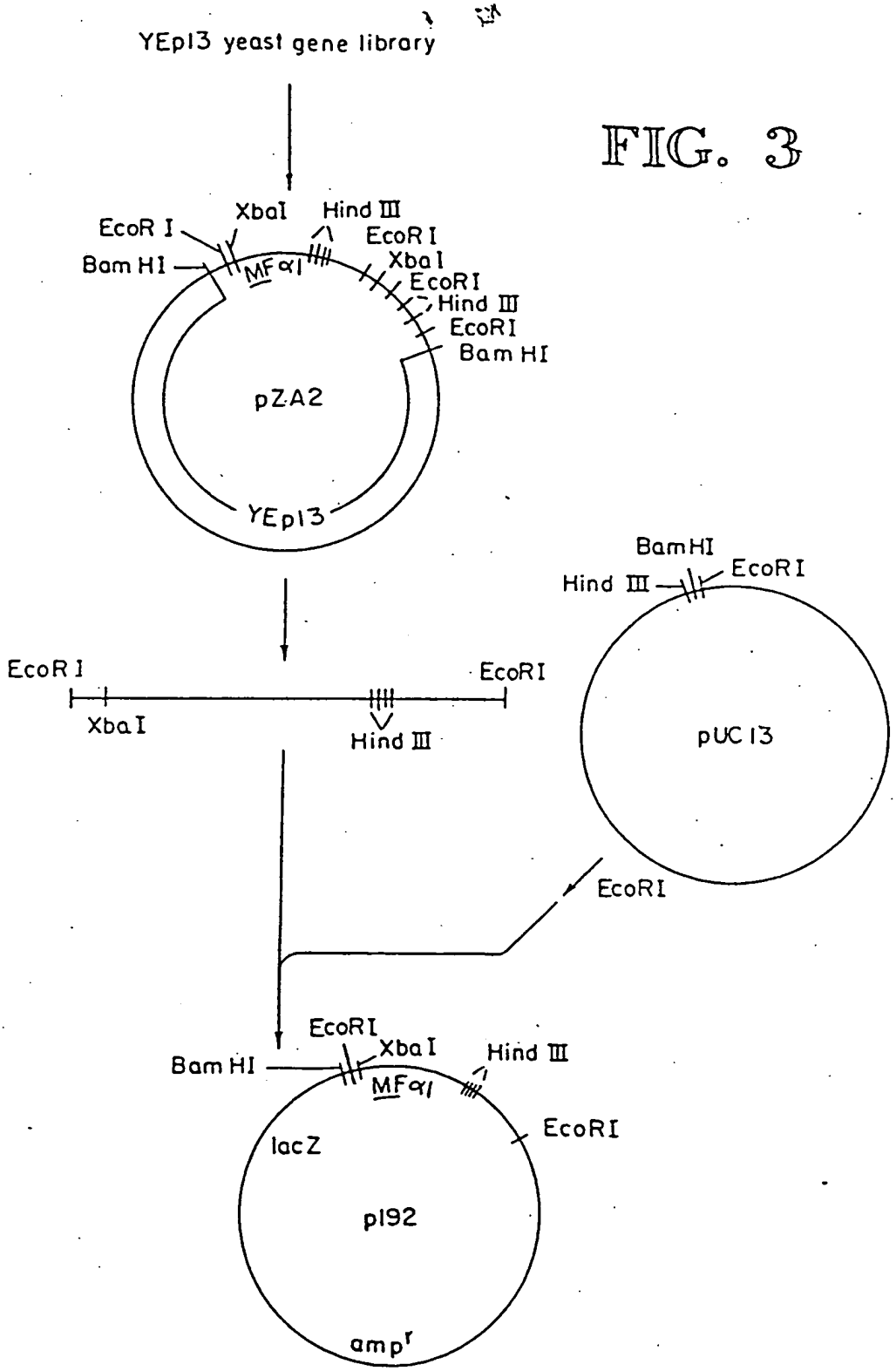
v-sis
Xba I
Xba I
EcoR I
pVS α
pUC 12

FIG. 2

FIG. 3


YEp13 yeast gene library
EcoR I
XbaI
Hind III
EcoR I
Bam HI
MFα1
Xba I
EcoRI
Hind III
EcoRI
Bam HI
pZA2
YEp13
EcoR I
EcoRI
XbaI
Hind III
BamHI
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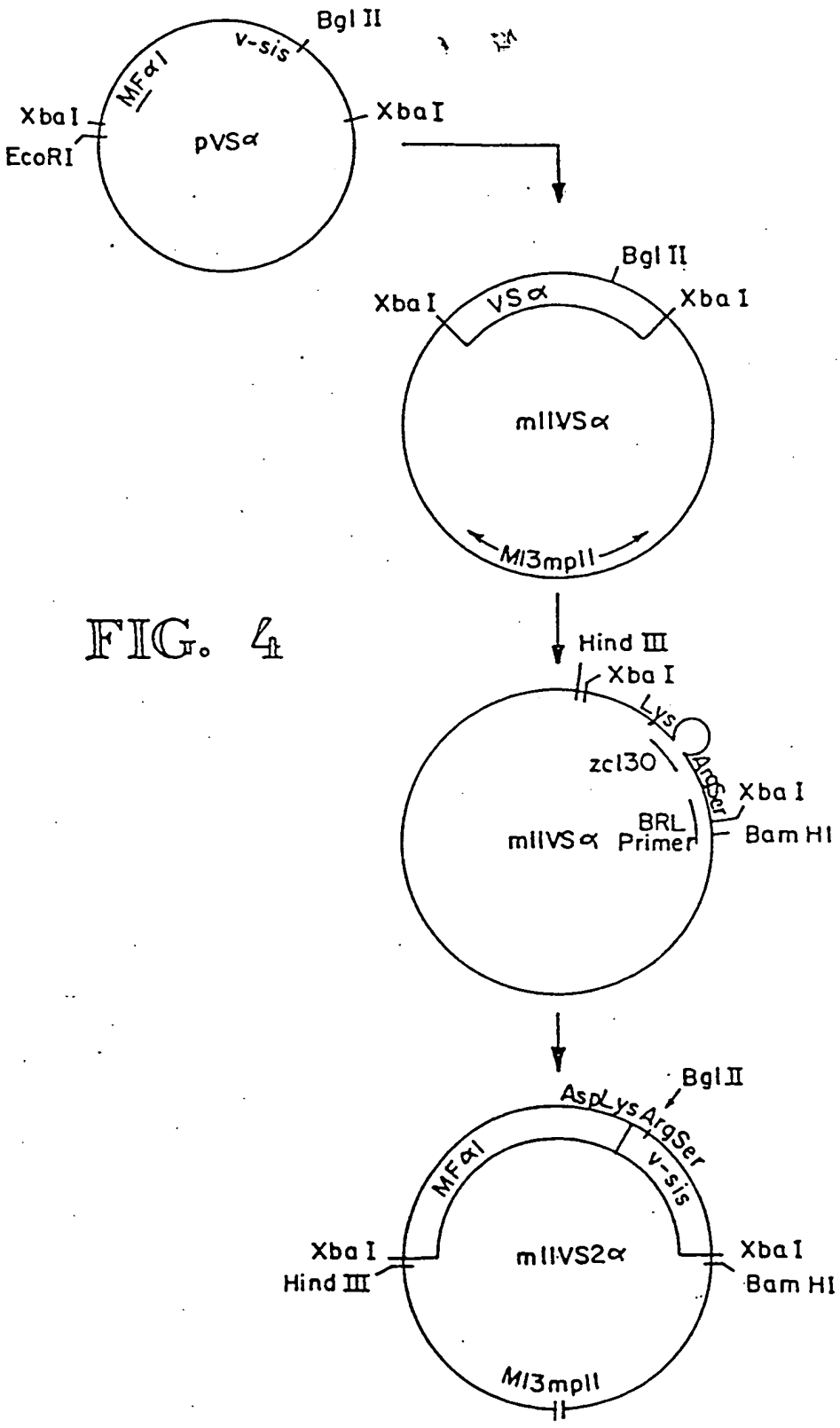
lacZ
p192
ampr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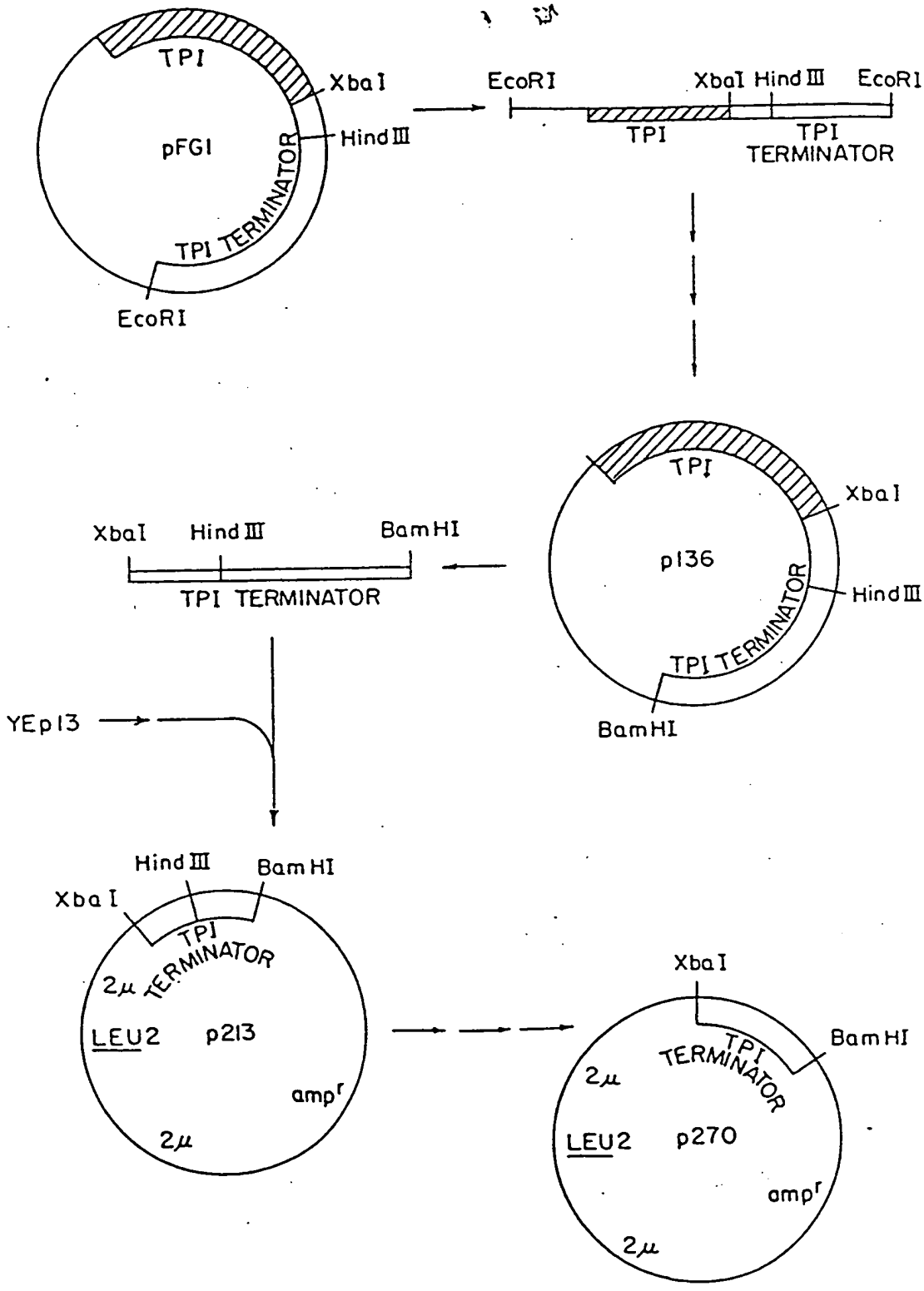
MFα1
Xba I
EcoRI
pVSα
Xba I
Bgl II
Xba I
VSα
Xba I
mIIVSα
M13mpII
Hind III
Xba I
Lys
zc130
ArgSer
Xba I
BRL
Primer
mIIVSα
Bam HI
Bgl II
Asp Lys Arg Ser
MFα1
v-sis
Xba I
Hind III
mIIVS2α
Xba I
Bam HI
M13mpII

FIG. 4

FIG. 5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EcoRI
EcoRI
XbaI HindIII
EcoRI
TPI
TPI
TERMINATOR
TPI
XbaI
p136
HindIII
TPI TERMINATOR
BamHI
XbaI
HindIII
BamHI
TPI TERMINATOR
YEp13
HindIII
BamHI
XbaI
TPI
TERMINATOR
2μ
LEU2
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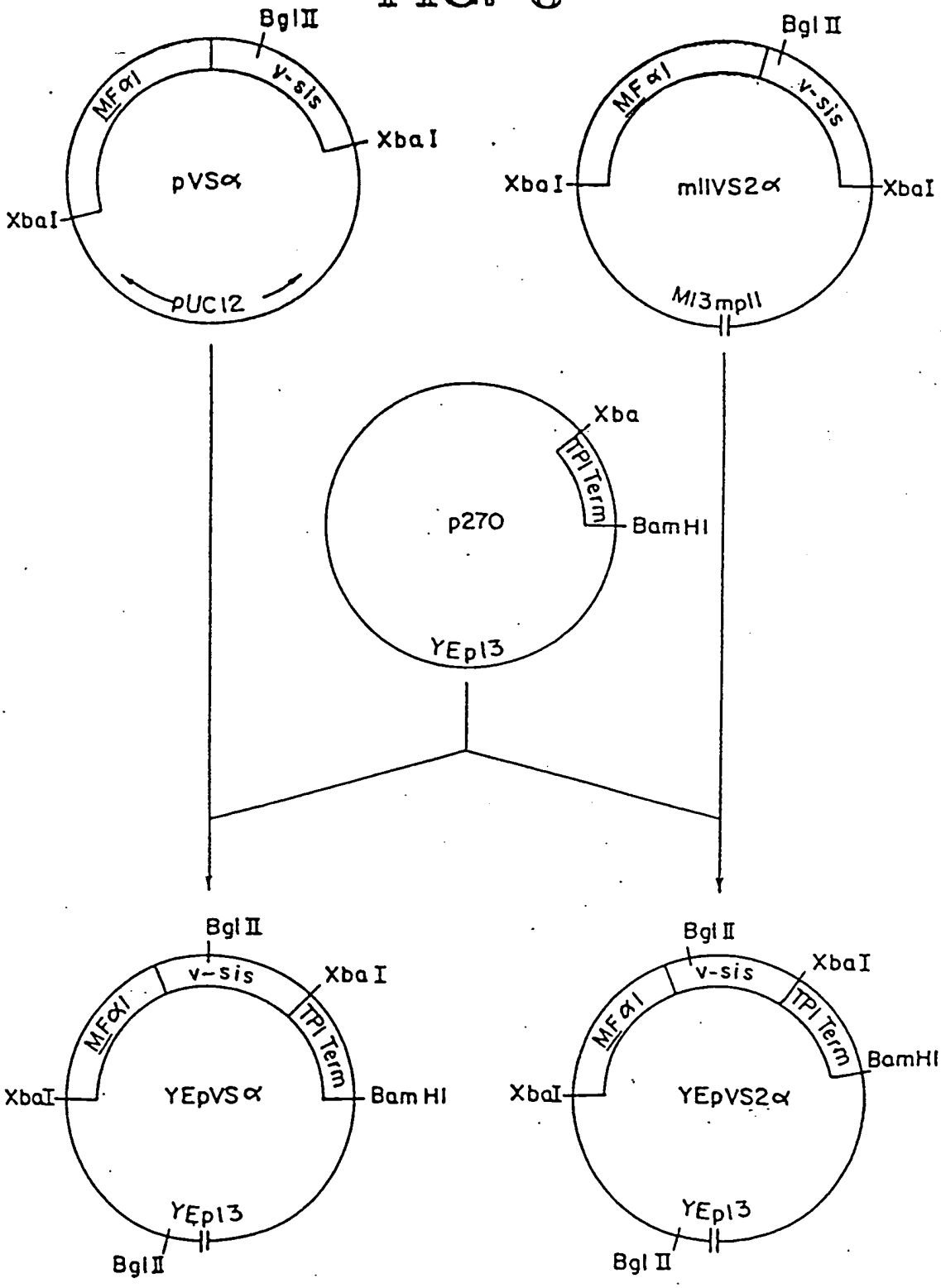
LEU2
p270
ampr
2μ

FIG. 6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XbaI
M13mpII
Xba
TPI Term
p270
BamHI
YEp13
BglII
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XbaI
MFα1
TPI Term
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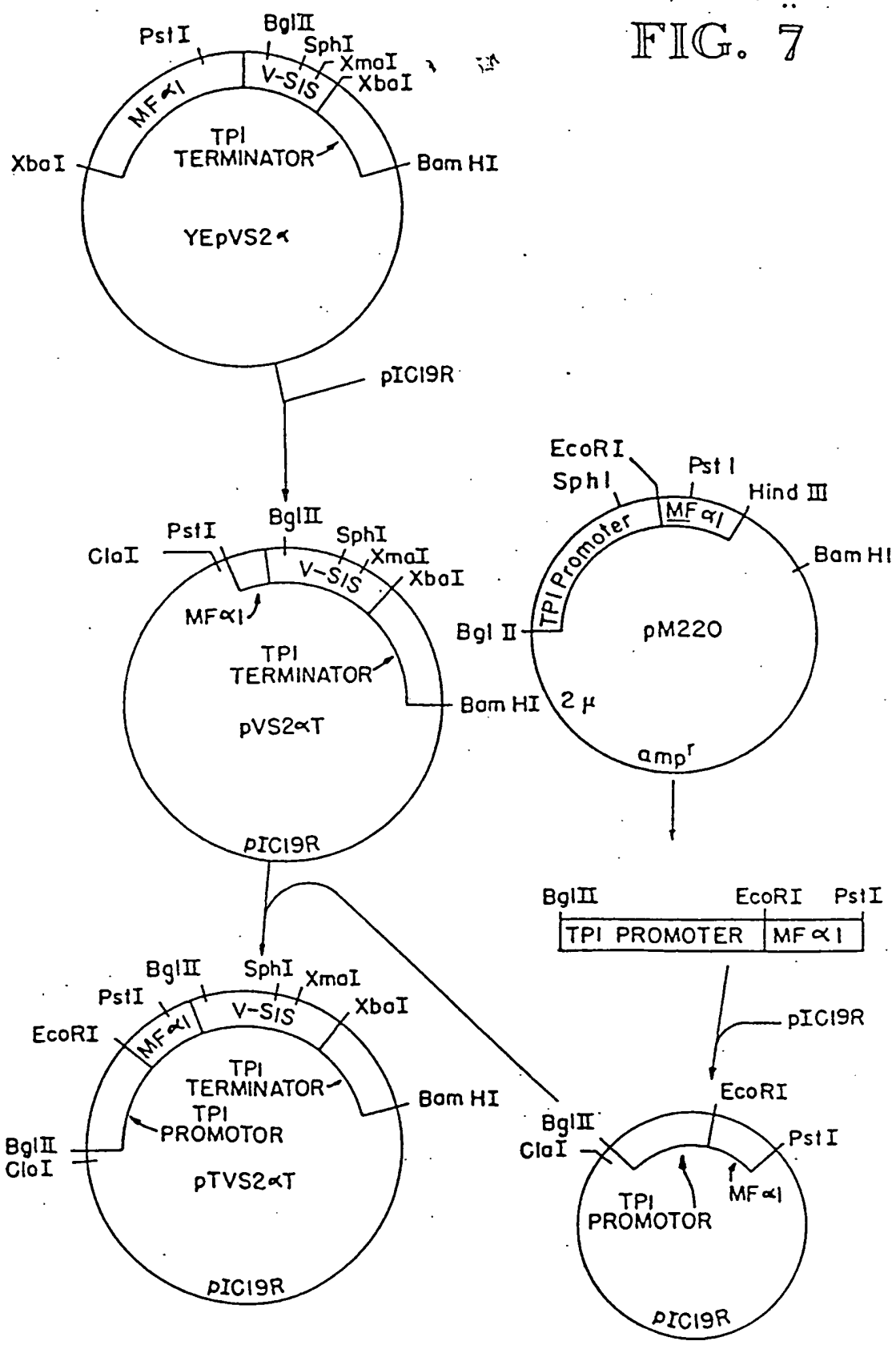
FIG. 7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SphI
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TPI TERMINATOR
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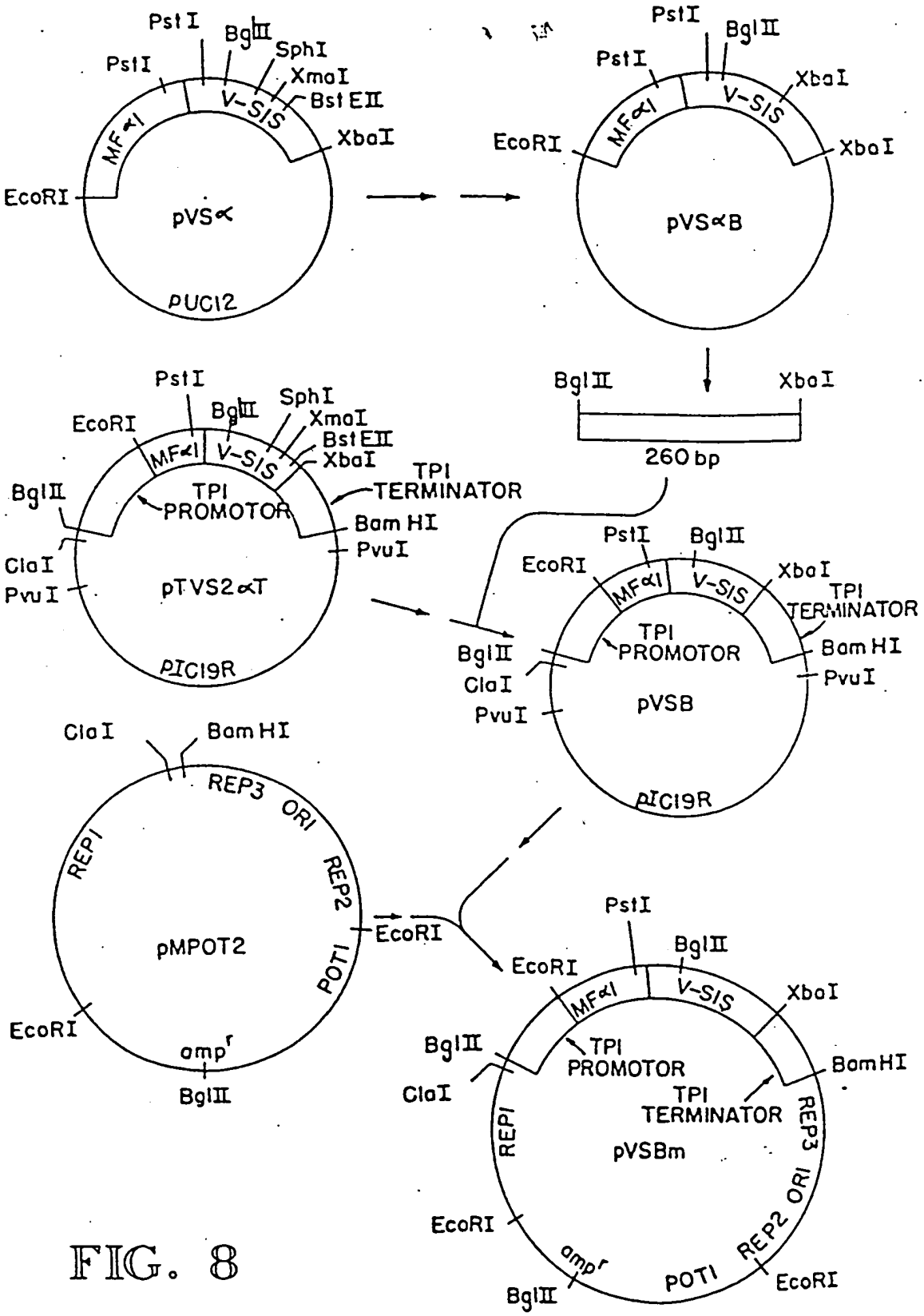
Bam HI
YEpVS2α
pIC19R
ClaI
PstI
BglII
SphI
XmaI
XbaI
MFα1
V-SIS
TPI TERMINATOR
Bam HI
pVS2αT
pIC19R
EcoRI
Sph I
Pst I
Hind III
TPI Promoter
MFα1
Bam HI
Bgl II
pM220
2μ
ampr
BglII
EcoRI
PstI
TPI PROMOTER
MFα1
pIC19R
BglII
SphI
XmaI
PstI
V-SIS
XbaI
EcoRI
MFα1
TPI TERMINATOR
Bam HI
TPI PROMOTOR
BglII
ClaI
pTVS2αT
pIC19R
EcoRI
BglII
PstI
ClaI
TPI PROMOTOR
MFα1
pIC19R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XmaI
BstEII
XbaI
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pUC12
pVSαB
260 bp
TPI
PROMOTOR
TERMINATOR
BamHI
PvuI
ClaI
pTVS2αT
pIC19R
pVSB
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ORI
REP1
REP2
POT1
pMPOT2
ampr
pVSBm

FIG. 8

FIG. 9

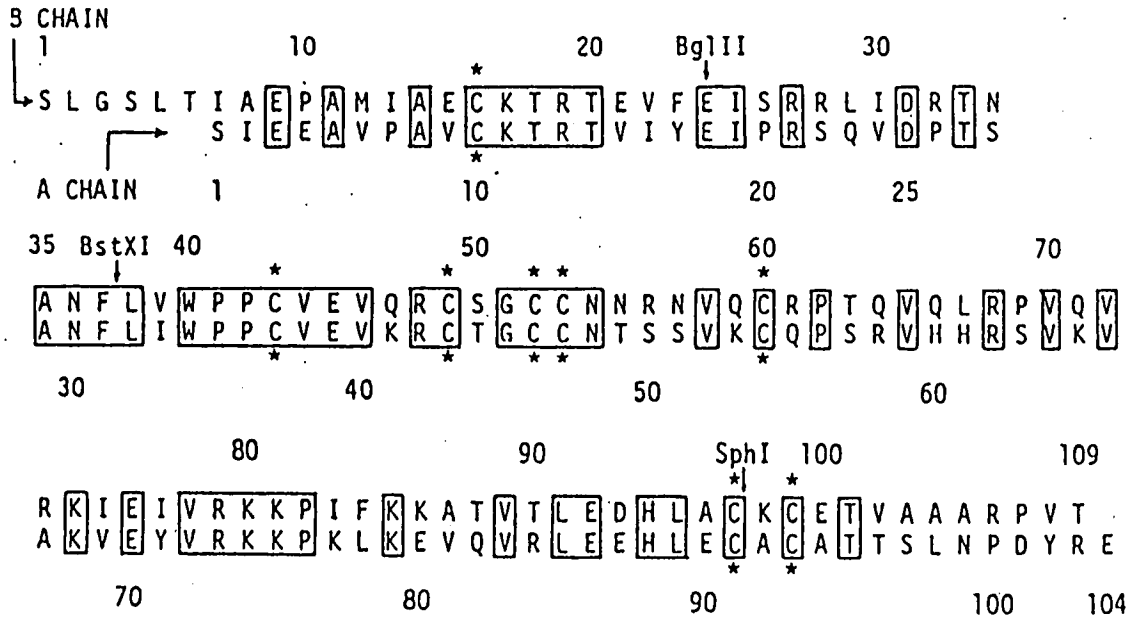
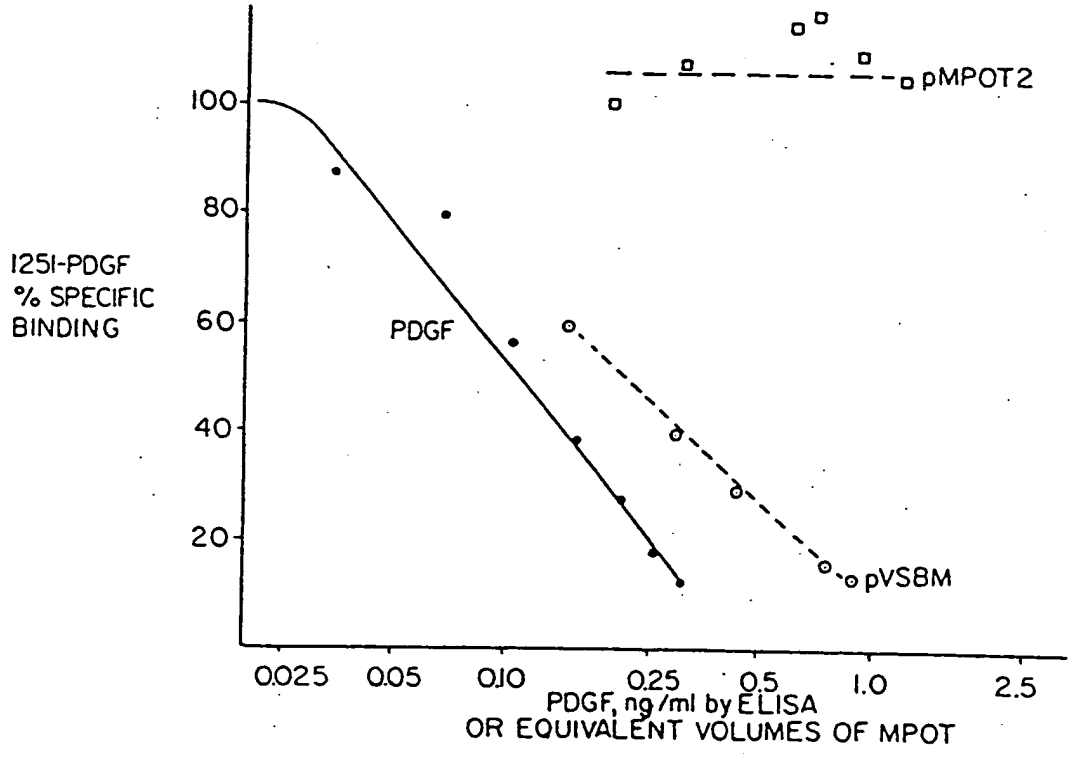


FIG. 10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100
80
60
40
20
125I-PDGF
% SPECIFIC
BINDING
PDGF
pVSBM
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0.05
0.10
0.25
0.5
1.0
2.5
PDGF, ng/ml by ELISA
OR EQUIVALENT VOLUMES OF MPOT

0259632

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 87 11 1591

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	BIOLOGICAL ABSTRACTS, vol. 81, no. 8, 1986, abstract no. 71311, Biological Abstracts, Inc., Philadelphia, P.A., US; J.D. KELLY et al.: "The B chain of platelet-derived growth factor alone is sufficient for mitogenesis", & EMBO (EUR. MOL. BIOL. ORGAN.) J 4(13A) 3399-3406, 1985[recd 1986] * Abstract * ---	1,5,7, 10,11, 12,16, 17,20	C 12 N 15/00 C 12 N 5/00 C 12 P 21/02 A 61 K 37/02 C 07 H 21/04
X	BIOLOGICAL ABSTRACTS, RRM, abstract no. 29014019, Philadelphia, US; E.W. RAINES et al.: "Biologic activity of platelet-derived growth factor-related sequences expressed in yeast saccharomyces-cerevisiae", & JOURNAL OF CELLULAR BIOCHEMISTRY SUPPLEMENT(US) 1985, vol. 0, no. 9, part A, p. 136 * Abstract * ---	1,11,12 ,15	
X	BIOLOGICAL ABSTRACTS/RRM, abstract no. 30041507, Philadelphia, US; K.H. SPRUGEL et al.: "Chemotactic activity of platelet derived growth factor-related sequences expressed in yeast", & JOURNAL OF CELL BIOLOGY(US), 1985, vol. 101, no. 5, part 2, p. 236A * Abstract * ---	1,11,12 ,15	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 12 N
X	EP-A-0 171 142 (ZYMOGENETICS) * Page 12; pages 28-36; claims * ---	1,5,7, 10-12, 16,17, 20	
X	EP-A-0 177 957 (ZYMOGENETICS) * Claims * -----	1-4,11, 12,16, 17,27, 33-35	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	23-11-1987	DELANGHE L.L.M.

CATEGORY OF CITED DOCUMENTS

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E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P0401)

European Patent Office

Application nu 87111591.

0259632

DECLARATION PURSUANT TO RULE 28, PARAGRAPH 4, OF THE EUROPEAN PATENT CONVENTION

The applicant has informed the European Patent Office that, until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, the availability of the micro-organism(s) identified below, referred to in paragraph 3 of Rule 28 of the European Patent Convention, shall be effected only by the issue of a sample to an expert.

IDENTIFICATION OF THE MICRO-ORGANISMS

Accession numbers of the deposits:

ATCC 20652, ATCC 20853, ATCC 39565, ATCC 20727, ATCC 39653